FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

7-06
4 October 2006

FINAL ASSESSMENT REPORT

APPLICATION A569

LIPASE FROM *HANSENULA POLYMORPHA* AS A PROCESSING AID (ENZYME)

For information on matters relating to this Assessment Report or the assessment process generally, please refer to http://www.foodstandards.gov.au/standardsdevelopment/

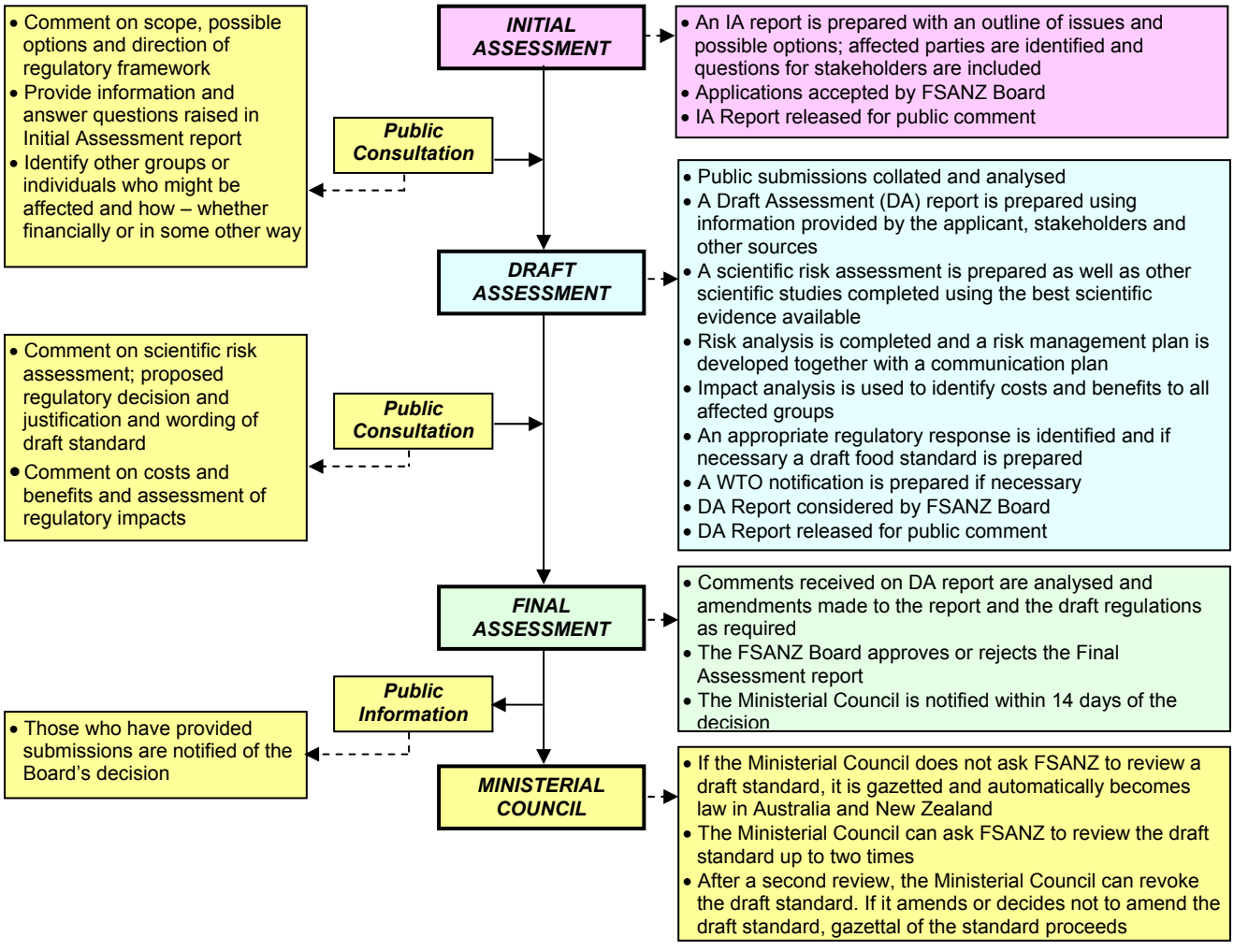
FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

FSANZ's role is to protect the health and safety of people in Australia and New Zealand through the maintenance of a safe food supply. FSANZ is a partnership between ten Governments: the Australian Government; Australian States and Territories; and New Zealand. It is a statutory authority under Commonwealth law and is an independent, expert body.

FSANZ is responsible for developing, varying and reviewing standards and for developing codes of conduct with industry for food available in Australia and New Zealand covering labelling, composition and contaminants. In Australia, FSANZ also develops food standards for food safety, maximum residue limits, primary production and processing and a range of other functions including the coordination of national food surveillance and recall systems, conducting research and assessing policies about imported food.

The FSANZ Board approves new standards or variations to food standards in accordance with policy guidelines set by the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council) made up of Australian Government, State and Territory and New Zealand Health Ministers as lead Ministers, with representation from other portfolios. Approved standards are then notified to the Ministerial Council. The Ministerial Council may then request that FSANZ review a proposed or existing standard. If the Ministerial Council does not request that FSANZ review the draft standard, or amends a draft standard, the standard is adopted by reference under the food laws of the Australian Government, States, Territories and New Zealand. The Ministerial Council can, independently of a notification from FSANZ, request that FSANZ review a standard.

The process for amending the *Australia New Zealand Food Standards Code* is prescribed in the *Food Standards Australia New Zealand Act 1991* (FSANZ Act). The diagram below represents the different stages in the process including when periods of public consultation occur. This process varies for matters that are urgent or minor in significance or complexity.



Final Assessment Stage

FSANZ has now completed two stages of the assessment process and held two rounds of public consultation as part of its assessment of this Application. This Final Assessment Report and its recommendations have been approved by the FSANZ Board and notified to the Ministerial Council.

If the Ministerial Council does not request FSANZ to review the draft amendments to the Code, an amendment to the Code is published in the *Commonwealth Gazette* and the *New Zealand Gazette* and adopted by reference and without amendment under Australian State and Territory food law.

In New Zealand, the New Zealand Minister of Health gazettes the food standard under the New Zealand Food Act. Following gazettal, the standard takes effect 28 days later.

Further Information

Further information on this Application and the assessment process should be addressed to the FSANZ Standards Management Officer at one of the following addresses:

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Assessment reports are available for viewing and downloading from the FSANZ website www.foodstandards.gov.au or alternatively paper copies of reports can be requested from FSANZ's Information Officer at info@foodstandards.gov.au including other general inquiries and requests for information.

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Executive Summary

FSANZ received an Application on 29 July 2005 from Axiome Pty Ltd acting as an agent for Danisco Australia Pty Ltd, to amend Standard 1.3.3 – Processing Aids of the Code to approve an enzyme, lipase triacylglycerol (EC number [3.1.1.3]), as a processing aid. Lipase triacylglycerol is produced, using recombinant DNA techniques, from the host yeast *Hansenula polymorpha* containing the gene coding for lipase triacylglycerol from the mould *Fusarium heterosporum*.

Processing aids are required to undergo a pre-market safety assessment before approval for use in Australia and New Zealand. There is currently approval for the use of different microbial and animal sources of lipase triacylglycerol in the Code.

Lipase triacylglycerol catalyses the hydrolysis of triglycerides, as well as phospholipids and galactolipids. It is claimed that the major application utilising the enzyme is in bread making, to improve dough stability and dough handling properties, and to improve the bread volume and crumb homogeneity. The enzyme can be used to treat egg yolk destined for baking or for the production of mayonnaise and salad dressing, and for the degumming of edible oils.

A safety assessment was required to determine whether the Code should be amended to permit the use of lipase triacylglycerol from the host yeast *H. polymorpha* containing the gene coding for lipase triacylglycerol isolated from the mould *F. heterosporum*.

The host organism, the yeast *H. polymorpha* is an approved host for the enzyme hexose oxidase which is listed in the Code. The genetic modification used to produce lipase triacylglycerol was produced using standard techniques and is well characterised.

The Safety Assessment Report concluded that:

- the production organism has a history of safe use as a production strain for food-grade enzyme preparations and has been shown not to produce toxic metabolites;
- the recombinant DNA in the production organism is considered stable and poses no safety concern;
- the enzyme preparation complies with international specifications;
- there was no evidence of toxicity in the acute toxicity study or in the sub-chronic toxicity study in rats;
- in a sub-chronic study in rats, the no-observed-adverse-effect-level (NOAEL) was 58,000 U/kg bw per day, which was the highest dose tested; and
- the enzyme preparation produced no evidence of genotoxic potential in *in vitro* assays in bacteria and in human peripheral blood lymphocytes.

From the available information, it is concluded that the use of this enzyme as a processing aid in food would not raise any public health and safety concerns.

The only regulatory options considered were to approve or not approve the use of lipase triacylglycerol produced from the host yeast *H. polymorpha*. Approval of the Application provides advantages to food manufacturers, mainly bread, noodle, pasta, baked goods and oil manufacturers and manufacturers who use eggs as emulsifiers. There should be no added costs to government regulators or consumers.

Public comment on the Initial Assessment Report was sought from 5 October 2005 to 16 November 2005. Seven submissions were received, of which two submitters did not support the Application, four supported or gave cautious support, while one submitter deferred comment to after the Draft Assessment. Public comment on the Draft Assessment Report was sought from 31 May 2006 to 12 July 2006. Seven submissions were received, with five supporting the Application and two opposing it. One issue was raised that has been addressed in this report.

The nomenclature of both *H. polymorpha* and *F. heterosporum* was assessed. An editorial note has been added to indicate that *H. polymorpha* is also called *Pichia angusta*.

The Final Assessment Report concludes that approval of lipase triacylglycerol, from the host yeast *H. polymorpha* containing the gene coding for lipase triacylglycerol isolated from the mould *F. heterosporum* as a processing aid is technologically justified and does not raise any public health and safety concerns.

FSANZ Decision

Approval is given for the enzyme lipase triacylglycerol from *Hansenula polymorpha* containing the gene coding for lipase triacylglycerol from *Fusarium heterosporum*. Permission is provided by adding this enzyme into the Table to clause 17 of Standard 1.3.3 – Processing Aids of the Code.

Statement of Reasons

The variation to Standard 1.3.3 – Processing Aids, giving approval for the use of lipase triacylglycerol from *H. polymorpha* containing the gene coding for lipase triacylglycerol from *F. heterosporum* as a processing aid, is recommended for the following reasons.

- Use of the enzyme is technologically justified since it has a role in bread manufacture to produce improved bread volume and crumb structure. The enzyme can also be used in other food applications such as baking, pasta and noodle manufacture, using egg yolk and whole eggs in food and in degumming of oil.

- A detailed safety assessment concluded that the use of the enzyme does not raise any public health and safety concerns.

- The variation to the Code is consistent with the section 10 objectives of the FSANZ Act. In particular, it does not raise any public health and safety concerns, the safety assessment has been based on the best available scientific evidence and it helps promote an efficient and internationally competitive food industry.

- Issues pertinent to the safety of the processing aid (enzyme) raised in submissions have been addressed.

- The regulatory impact assessment has concluded that the benefits of permitting the use of the enzyme outweigh any costs associated with its use.

- The most cost-effective means to achieve what the Application seeks, namely permissions to use lipase triacylglycerol from *Hansenula polymorpha* containing the gene coding for lipase triacylglycerol from *Fusarium heterosporum* as a processing aid, is a variation to Standard 1.3.3.

1. Introduction

FSANZ received an Application on 29 July 2005, from Danisco Australia Pty Ltd (submitted by Axiome Pty Ltd), to amend Standard 1.3.3 – Processing Aids of the Code to approve an enzyme, lipase triacylglycerol (EC number [3.1.1.3]), as a processing aid. Lipase triacylglycerol is produced, using recombinant DNA techniques, from the host yeast *H. polymorpha* containing the gene coding for lipase triacylglycerol from the mould *F. heterosporum*.

The Applicant claims lipase triacylglycerol catalyses the hydrolysis of triglycerides, as well as phospholipids and galactolipids. It is claimed that the major application utilising the enzyme is in bread making, to improve dough stability and dough handling properties, and to improve the bread volume and crumb homogeneity. The Applicant also claims the enzyme can be used to treat egg yolk destined for baking or for the production of mayonnaise and salad dressing, and for the degumming of edible oils.

2. Regulatory Problem

Processing aids are required to undergo a pre-market safety assessment before approval for use. A processing aid is a substance used in the processing of raw materials, foods or ingredients, to fulfil a technological purpose relating to treatment or processing, but does not perform a technological function in the final food.

The Table to clause 17 of Standard 1.3.3 contains a list of permitted enzymes of microbial origin. There are a number of approved sources of the enzyme, lipase triacylglycerol, but not the source *H. polymorpha* containing the gene coding for lipase triacylglycerol from the mould *F. heterosporum*. The yeast *H. polymorpha* is an approved host for a genetically modified (GM) source organism of the hexose oxidase enzyme, being the source *H. polymorpha,* containing the gene for hexose oxidase isolated from *Chondrus crispus*.

3. Objective

The objective of this assessment is to determine whether it is appropriate to amend the Code to permit the use of lipase triacylglycerol from *H. polymorpha* containing the gene coding for lipase triacylglycerol from *F. heterosporum.*

In developing or varying a food standard, FSANZ is required by its legislation to meet three primary objectives which are set out in section 10 of the FSANZ Act. These are:

- the protection of public health and safety;
- the provision of adequate information relating to food to enable consumers to make informed choices; and
- the prevention of misleading or deceptive conduct.

In developing and varying standards, FSANZ must also have regard to:

- the need for standards to be based on risk analysis using the best available scientific evidence;
- the promotion of consistency between domestic and international food standards;
- the desirability of an efficient and internationally competitive food industry;
- the promotion of fair trading in food; and
- any written policy guidelines formulated by the Ministerial Council.

4. Background

4.1 Historical Background

Lipases have a large number of uses both in the food industry as well as in the broader biotechnology area[1].

In the food industry, lipases have a number of uses, which have increased in the last few years. They can be used in the fruit juice industry, baked goods, vegetable fermentation and dairy industries. Lipases have traditionally been used in the oils and fats industries where lipases catalyse the cleavage of fatty acids from triglycerides in fats. Lipases can be used for de-gumming purposes in the fats and oils industries. They can also be used to improve the emulsifying properties of ingredients (such as lecithin and egg yolk) during food processing.

Lipase triacylglycerol (EC number [3.1.1.3]) is currently approved as an enzyme with different microbial sources in the Table to clause 17 – Permitted enzymes of microbial origin of Standard 1.3.3. It is also listed in the Table to clause 15 – Permitted enzymes of animal origin, as Lipase (EC [3.1.1.3]), being sourced from bovine stomach; salivary glands or forestomach of calf, kid or lamb; porcine or bovine pancreas.

There is another different lipase listed in Table to clause 17 of Standard 1.3.3, called lipase, monoacylglycerol with EC number of [3.1.1.23].

5. Relevant Issues

5.1 Nature and technological justification of the enzyme

In the Table to clause 17 – Permitted enzymes of microbial origin of Standard 1.3.3 of the Code the name of this enzyme of this Application is lipase triacylglycerol.

The systematic name of the enzyme is triacylglycerol acylhydrolase, while the common name is triacylglycerol lipase[2]. Other names include lipase, triglyceride lipase and tributyrase.

[1] Pandey, A.; Benjamin, S.; Soccol, C.R.; Nigam, P.; Krieger, N. and Soccol, V.T. (1999) The realm of microbial lipases in biotechnology, *Biotechnol. Appl. Biochem.* **29**, 119-131.
[2] International Union of Biochemistry and Molecular Biology (IUBMB) Enzyme Nomenclature http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/1/1/32.html, accessed on 31 March 2006

It has the Enzyme Commission (EC) number of [3.1.1.3] and a Chemical Abstracts Service (CAS) number of 9001-62-1.

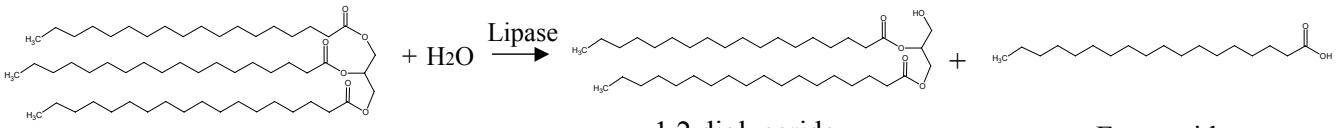
The enzyme preparation is an off-white to brownish coloured powder which is freely soluble in water. The enzyme is stable between pH 5 and 7 with optimum pH stability at 6.5. The enzyme activity occurs between pH 4 to 10, with its optimum activity at pH 8. The optimum temperature of use is approximately 40ºC. It is not thermally stable above 45ºC in an aqueous solution. The molecular weight of the enzyme was determined to be 30 kDa by the SDS-PAGE gel method.

Lipases are enzymes that catalyse the cleavage of triglycerides to fatty acids. The enzyme is characterised by its ability to catalyse the reaction:

Triacylglycerol + H_2O → Diacylglycerol + a fatty acid anion (a carboxylate)

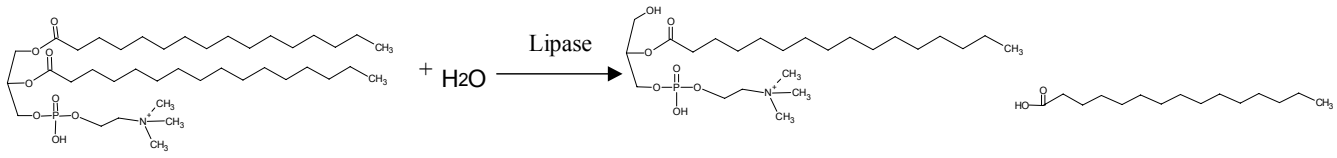
The enzyme lipase triacylglycerol can also hydrolyse phospholipids and galactolipids.

The Application states that the enzyme hydrolyses the ester bonds primarily in the 1 and 3 positions of the triglyceride molecule. It also contains evidence that the enzyme has specificity towards hydrolysing fatty acids from sn-1 (position 1) of phospholipids and galactolipids, in dough. The following schematics indicating how the lipase enzyme reacts with triglycerides, phospholipids and galactolipids has been taken from the Application.



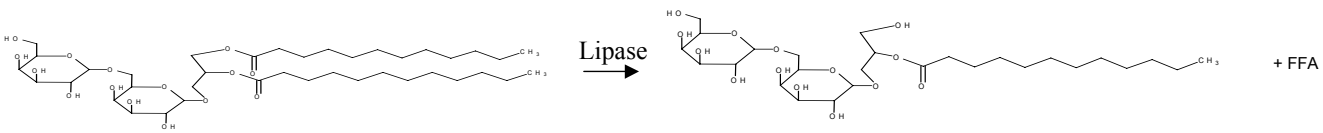
Triglyceride 1,2 diglyceride Fatty acid

The enzyme also has activity towards sn-1 ester bonds in other lipid components including diacyl-phospholipids, e.g.:



Phosphatidylcholine Lyso-phosphatidylcholine Fatty acid

and diacyl-galactolipids, e.g.:



Digalactosyl diglyceride Digalactosyl monoglyceride

The lipase triacylglycerol enzyme preparation is produced by submerged fermentation using a selected strain of the yeast *H. polymorpha* that has the gene coding for lipase triacylglycerol isolated from *F. heterosporum* inserted by recombinant DNA techniques. After fermentation is completed the biomass is removed by centrifugation and filtration. The supernatant fermentation broth which contains the enzyme is filtered and then concentrated by ultra-filtration. The ultra-filtrate is then sterile filtered and finally spray dried or granulated onto a food grade carrier such as wheat starch. The manufacturing process is that commonly used to produce enzymes from microbial sources.

It is unlikely that there are any dietary or nutrition implications with this Application. The enzyme is to be used as a processing aid and the majority of the enzyme will be removed from the final product as part of the manufacturing process. Some small proportion of the enzyme may remain in the final product but it will have been inactivated to a protein, having the same nutritional value as protein. The enzyme will be used at very low levels. Enzymes and their reaction by-products, diacylglycerol and fatty acids, are natural components of food and no different to other constituents of food.

The Applicant tested the activities of the lipase enzyme against a number of other substrates rather than various lipids to assess any extra activity. The results were negative for alpha-amylase, endo-xylanase, protease, glucose oxidase and beta-glucanase, indicating that the enzyme has reasonable purity with no unintended activity to other substrates.

5.2 Safety assessment

The enzyme is used as a processing aid only, and is not expected to be present in the final food. Any residue would be in the form of an inactivated enzyme, which would be metabolised like any other protein.

The Safety Assessment Report (**Attachment 2**) concluded that:

- the production organism has a history of safe use as a production strain for food-grade enzyme preparations and has been shown not to produce toxic metabolites;
- the recombinant DNA in the production organism is considered stable and poses no safety concern;
- the enzyme preparation complies with international specifications;
- there was no evidence of toxicity in the acute toxicity study or in the sub-chronic toxicity study in rats;
- in a sub-chronic study in rats, the no-observed-adverse-effect-level (NOAEL) was 58,000 U/kg bw per day, which was the highest dose tested; and
- the enzyme preparation produced no evidence of genotoxic potential in *in vitro* assays in bacteria and in human peripheral blood lymphocytes.

From the available information, it is concluded that the use of this enzyme as a processing aid in food would not raise any public health and safety concerns.

5.3 Other international regulatory standards

The same enzyme from the same Applicant was deemed self-affirmed GRAS in the USA on 27 May 2005 (the summary report of the GRAS Expert panel is contained in the Application).

The Applicant claims the enzyme preparation complies with the specifications for enzyme preparations in the Food Chemicals Codex, 5th Edition, 2004[3] and JECFA Compendium of Food Additive Specifications, Volume 1, Annex 1, Addendum 9 2001, (General Specifications and Considerations for Enzyme Preparations Used in Food Processing)[4].

5.4 Issues arising from submissions

Two types of issues came out of public submissions to the Initial and the Draft Assessment Reports. The first relates to the GM aspects, and is discussed below in section 5.4.1. The second relates to the source organism nomenclature, and as such is discussed under this heading, in section 5.5.4.

5.4.1 GM aspects

Two submitters objected, at both Initial and Draft Assessments, to the Application on grounds of the GM aspects of the Application. No other issues were raised, beyond an opposition to GM food or food containing components derived using GM techniques.

5.4.2 FSANZ response

The labelling requirements for genetically modified foods in Standard 1.5.2 of the Code are among the most comprehensive labelling requirements in the world. They were written following extensive public consultation and represent a fair balance between what industry and consumers want and what governments can enforce. They are not there for any safety concern, but rather to allow consumers to purchase or avoid products depending on their own beliefs.

The relevant section of the Code relating to labelling of genetically modified food is contained in Division 2 – Labelling etc of food produced using gene technology of Standard 1.5.2. This requires that processing aids (and food additives) be labelled where novel DNA and/or novel protein from the processing aid or food additive remains present in food.

In the case of enzymes produced from genetically modified (GM) micro-organisms, this enzyme is not a novel protein since it is identical to other enzymes sourced from non-GM sources. The refinement process for the enzyme preparation removes all the source organism from the preparation so there is no *novel* DNA in the enzyme preparation. Small amounts of enzymes (inactivated or not) from a genetically modified source remaining in food do not require labelling under the gene technology labelling requirements because they are not considered to be 'novel'.

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[3] Food Chemicals Codex (2004), National Academy of Sciences, Food and Nutrition Board, Committee on Food Chemical Codex, 5th Edition, National Academy Press, Washington DC, pp 146-152.

[4] Joint FAO/WHO Expert Committee on Food Additives (JECFA) (2001). General specifications and considerations for enzyme preparations used in food processing. FAO Food and Nutrition Paper 52, Addendum 9, pp 37-39.

This is the case for a number of enzymes sourced from GM micro-organisms in the Code. Therefore foods produced using this enzyme will not be required to be labelled as genetically modified.

5.5 Source organism nomenclature

To ensure that the names of the two microbes are correct as used by the Applicant, a literature search was conducted. The research also involved internet database scans. The results are summarised below.

5.5.1 Hansenula polymorpha

The databases of The American Type Culture Collection (ATCC)[5], Centraalbureau voor Schimmelcultures (CBS)[6] and National Collection of Yeast Cultures (NCYC)[7] were searched for references to *H. polymorpha.*

When searched for *H. polymorpha*, the ATCC webpage produced 44 entries. It was notable that for the entries, the descriptions stated '*Pichia angusta*, deposited as *Hansenula polymorpha*'.

A search of the filamentous fungi database on the CBS webpage brought up one record for *H. polymorpha*, with nine strains listed as being available for this record. The record states that *H. polymorpha* is a synonym of *Pichia angusta*, and all nine strains registered under the name *Pichia angusta*.

The NCYC database did not contain any listings for *H. polymorpha*, with the only record for that genus being for *H. misumaiensis*. In contrast, a search for *Pichia angusta* produced one complete record. Like the ATCC records, the strain was recorded as being deposited as *H. polymorpha*.

Kurtzman and Fell, in their text, 'The Yeasts, A Taxonomic Study'[8], state in the index of taxa by genus and species that: 'the names of the genera, species and varieties accepted by the authors are indicated in bold type'. *Hansenula polymorpha* is listed in normal type, whilst *Pichia angusta* is in bold type. The reference for *Pichia angusta* states that *Hansenula polymorpha* is a synonym. The respective dates for the names are 1984 for *Pichia angusta* and 1959 for *Hansenula polymorpha*. The comments in the listing indicate that those yeast species with hat-shaped ascospores were transferred from *Hansenula* to *Pichia*.

A paper from Kurtzman[9] indicates that a study of DNA relatedness between phenotypically similar species led to the transfer of hat-spored species of *Hansenula* to *Pichia*.

[5] American Type Culture Collection see: http://www.atcc.org
[6] Centraalbureau voor Schimmelcultures, Institute of the Royal Netherlands Academy of Arts and Sciences see: http://www.cbs.knaw.nl
[7] National Collection of Yeast Cultures see: http://www.ncyc.co.uk/
[8] Kurtzman, C.P. and Fell. J.W. (1998) (eds) *The Yeasts: A Taxonomic Study,* 4th ed, Elsevier Science, Amsterdam.
[9] Kurtzman, C.P. (1984) Synonomy of the yeast genera Hansenula and Pichia demonstrated through comparisons of deoxyribonucleic acid relatedness. Antonie Van Leeuwenhoek, 50(3): 209-17.

Dr Ailsa Hocking, Section Leader, Mycology and Mycotoxins, Food Science Australia, has previously indicated that the CBS database and the text by Kurtzman and Fell were reliable and taxonomically trustworthy references.

Barnett *et al.*[10] lists *Pichia angusta* as the recognised current name for this organism with *Hansenula angusta* being the basionym. The basionym, or first name validly published, was dated 1959, with the change to *Pichia angusta* occurring in 1984. This is in agreement with the information from Kurtzman and Fell.

5.5.2 Fusarium heterosporum

The databases of ATCC, CBS, the German National Resource Centre for Biological Material (DSMZ)[11] and NCYC were searched for references to *heterosporum.*

The ATCC contained 13 references for *F. heterosporum.* No synonyms were listed.

The CBS database contained two records for *F. heterosporum.* No synonyms were listed for the name.

The DSMZ contained one record for *F. heterosporum* in the filamentous fungi database. No synonyms were listed.

The NCYC database did not contain any references to *F. heterosporum.*

There was no reference to *F. heterosporum* in Kurtzman and Fell.

5.5.3 Conclusion

The evidence would suggest that *H. polymorpha* is not the current accepted name for this yeast, and that it would be more correctly referred to as *Pichia angusta*. FSANZ considered this issue and proposes to use the nomenclature that the Applicant has used in their Application. This is because the Safety Assessment Report (Attachment 2) was performed on *H. polymorpha*, which is also the name the Applicant uses internationally and which has been approved with this name by other regulatory agencies. An earlier application (A475 – Hexose Oxidase as a Processing Aid (Enzyme)) for another enzyme (hexose oxidase) by the same Applicant was approved and then gazetted into the Code also uses the term *H. polymorpha* as the host organism.

FSANZ therefore will add an editorial note following the Table to clause 17 in Standard 1.3.3 to the effect that *H. polymorpha* is also known as *Pichia angusta*.

There was no evidence to suggest that *F. heterosporum* is not a current accepted name for this mould.

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[10] Barnett, J.A, Payne, R.W. and Yarrow, D. (2000) *Yeasts: Characteristics and Identification,* 3rd edition, Cambridge University Press, UK.

[11] German National Resource Centre for Biological Material (DSMZ) see: http://www.dsmz.de/species/sp30053.htm

5.5.4 Nomenclature issue from submission

5.5.4.1 The issue

A submission has raised a statement regarding the source organism nomenclature, suggesting that the nomenclature needs to be revisited. The submitter advised that the source organism be referred to as *Pichia angusta*.

5.5.4.2 FSANZ response

FSANZ will keep the organism nomenclature as *H. polymorpha* for the reasons described above in 5.5.3 but will add an editorial note following the Table to clause 17 in Standard 1.3.3 to indicate that *H. polymorpha* is also known as *Pichia angusta*.

6. Regulatory Options

FSANZ is required to consider the impact of various regulatory (and non-regulatory) options on all sectors of the community, which includes consumers, food industries and governments in Australia and New Zealand. The benefits and costs associated with the proposed amendment to the Code have been analysed using regulatory impact principles.

The two regulatory options available for this Application are:

Option 1. **Not approve** the use of lipase triacylglycerol from *H. polymorpha* containing the gene coding for lipase triacylglycerol from *F. heterosporum* as a processing aid.

Option 2. **Approve** the use of lipase triacylglycerol from *H. polymorpha* containing the gene coding for lipase triacylglycerol from *F. heterosporum* as a processing aid.

7. Impact Analysis

7.1 Affected Parties

The affected parties to this Application include the following:

1. those sectors of the food industry wishing to produce and market food products manufactured using this enzyme;

2. consumers; and

3. Australian, State, Territory and New Zealand Government agencies that enforce food regulations.

7.2 Impact Analysis

7.2.1 Option 1 – Status quo

There are no perceived benefits to industry, government regulators or consumers if this option is taken.

There are disadvantages to those food industries, specifically bread and bakery manufacturers, if this option is taken, since they will not have an enzyme available to them that may improve their process efficiencies.

7.2.2 Option 2 – Approve the enzyme

There are advantages to bread manufacturers, to improve dough stability and dough handling properties, and to improve the bread volume and crumb homogeneity. The Applicant also claims the enzyme can be used to treat egg yolk destined for baking or for the production of mayonnaise and salad dressing, and for the degumming of edible oils.

There should be no added costs to government food regulators or consumers.

Option 2, which supports the approval of lipase triacylglycerol from *H. polymorpha* containing the gene coding for lipase triacylglycerol from *F. heterosporum* as a processing aid is the preferred option, since it has advantages for the food industry but has no significant cost for government regulators, consumers or food manufacturers.

8. Consultation

8.1 Public consultation

Public comment on the Initial Assessment Report for this Application was sought from 5 October until 16 November 2005. Seven submissions were received of which four supported the Application, one reserved comment until the Draft Assessment, and two opposed the Application with their opposition due to the GM aspects of the Application. Public comment on the Draft Assessment Report for this Application was sought from 31 May until 12 July 2006. Seven submissions were received of which five supported the Application, and two opposed the Application with their opposition due to the GM aspects of the Application. **Attachment 4** summarises the submissions received during both Initial and Draft round of public comments.

8.2 World Trade Organization (WTO)

As members of the World Trade Organization (WTO), Australia and New Zealand are obligated to notify WTO member nations where proposed mandatory regulatory measures are inconsistent with any existing or imminent international standards and the proposed measure may have a significant effect on trade.

Amending the Code to approve the enzyme lipase triacylglycerol from *H. polymorpha* containing the gene coding for lipase triacylglycerol from *F. heterosporum* as a processing aid is unlikely to have a significant effect on trade. Most countries do not regulate enzymes as processing aids as in Australia and New Zealand. Also since the enzyme is considered a processing aid there is no requirement to label final food for the presence of the enzyme. The enzyme preparation is consistent with the international specifications for food enzymes of Food Chemicals Codex (5th Edition, 2004) and JECFA. It was determined that there was no need to notify the WTO under either the Technical Barrier to Trade (TBT) or Sanitary and Phytosanitary Measure (SPS) Agreements.

9. The Decision

FSANZ has prepared a variation to the Table to clause 17 of Standard 1.3.3 to permit the use of the enzyme lipase triacylglycerol from *H. polymorpha* containing the gene coding for lipase triacylglycerol from *F. heterosporum*.

The variation to Standard 1.3.3 – Processing Aids, giving approval for the use of lipase triacylglycerol from *H. polymorpha* containing the gene coding for lipase triacylglycerol from *F. heterosporum* as a processing aid, is recommended for the following reasons.

- Use of the enzyme is technologically justified since it has a role in bread manufacture to produce improved bread volume and crumb structure. The enzyme can also be used in other food applications such as baking, pasta and noodle manufacture, using egg yolk and whole eggs in food and in degumming of oil.

- A detailed safety assessment concluded that the use of the enzyme does not raise any public health and safety concerns.

- The variation to the Code is consistent with the section 10 objectives of the FSANZ Act. In particular, it does not raise any public health and safety concerns, the safety assessment has been based on the best available scientific evidence and it helps promote an efficient and internationally competitive food industry.

- Issues pertinent to the safety of the processing aid (enzyme) raised in submissions have been addressed.

- The regulatory impact assessment has concluded that the benefits of permitting the use of the enzyme outweigh any costs associated with its use.

- The most cost-effective means to achieve what the Application seeks, namely permissions to use lipase triacylglycerol from *Hansenula polymorpha* containing the gene coding for lipase triacylglycerol from *Fusarium heterosporum* as a processing aid, is a variation to Standard 1.3.3.

ATTACHMENTS

1. Draft variation to the *Australia New Zealand Food Standards Code*
2. Safety Assessment Report
3. Food Technology Report
4. Summary of Submissions

Attachment 1

DRAFT VARIATION TO THE *AUSTRALIA NEW ZEALAND FOOD STANDARDS CODE*

To commence: On gazettal

[1] ***Standard 1.3.3*** *of the Australia New Zealand Food Standards Code is varied by –*

[1.1] *inserting in the* Table to clause 17, *for the enzyme* Lipase, triacylglycerol EC [3.1.1.3], *the source –*

Hansenula polymorpha, containing the gene for Lipase, triacylglycerol isolated from *Fusarium heterosporum*

[1.2] *inserting in the* Editorial note *following the* Table to clause 17 –

Hansenula polymorpha is also known as *Pichia angusta.*

Attachment 2

Safety Assessment Report

A569 – LIPASE FROM *HANSENULA POLYMORPHA* AS A PROCESSING AID (ENZYME)

Summary and Conclusion

Application A569 seeks approval for the use of a lipase triacylglycerol enzyme preparation, (referred to as KLM1 by the Applicant and used by that name in this report as an abbreviation), as a processing aid, mainly in the manufacture of bread and bakery products. The enzyme is produced by fermentation using a selected yeast strain *Hansenula polymorpha,* containing the gene coding for lipase triacylglycerol from *Fusarium heterosporum*.

The enzyme is used as a processing aid only, and is not expected to be present in the final food. Any residue would be in the form of inactivated enzyme, which would be metabolised like any other protein.

The safety assessment concluded that:

- the production organism has a history of safe use as a production strain for food-grade enzyme preparations and has been shown not to produce toxic metabolites;
- the recombinant DNA in the production organism is considered stable and poses no safety concern;
- the enzyme preparation complies with international specifications;
- there was no evidence of toxicity in the acute toxicity study or in the sub-chronic toxicity study in rats;
- in the sub-chronic study in rats, the no-observed-adverse-effect-level (NOAEL) was 58,000 U/kg bw per day, which was the highest dose tested; and
- the enzyme preparation produced no evidence of genotoxic potential in *in vitro* assays in bacteria and in human peripheral blood lymphocytes.

From the available information, it is concluded that the use this enzyme as a processing aid in food would not raise any public health and safety concerns.

1. Introduction

Application A569 seeks approval for the use of the lipase enzyme KLM1 produced by fermentation of a selected yeast strain *H. polymorpha*. The systematic name of the principal enzyme activity is triacylglycerol acylhydrolase (EC 3.1.1.3, CAS No. 9001-62-1). Other names include lipase, triglyceride lipase and tributyrase. The enzyme preparation has the marketing name of GRINDAMYL™ POWERBake.

The production organism, *H. polymorpha,* contains a lipase encoding gene derived from the mould *F. heterosporum*, which is unsuitable itself for commercial production of the enzyme.

The Applicant seeks permission for the use of the KLM1 enzyme preparation as a processing aid in the manufacture of bread, pasta and noodles, in egg yolk and whole eggs and in the degumming of oil. The enzyme is not expected to be present in the final food. Any residue would be in the form of inactivated enzyme, which would be metabolised like any other dietary protein.

Numerous studies relevant for a safety assessment were submitted in support of this Application, including (i) an acute oral toxicity study in rats; (ii) a 90 day subchronic oral toxicity study in rats; (iii) a reverse mutation test in bacteria; and (iv) a mammalian chromosome aberration test.

2. History of use

KLM1 is produced by fermentation of a recombinant strain of the yeast *H. polymorpha*, which contains a gene encoding the lipase enzyme derived from *F. heterosporum*.

2.1 Safety of the donor organism

The donor organism is a mould, *F. heterosporum* (CBS 782.83). Fusarium species are commonly found in soil and are pathogenic in plants. *F. heterosporum* (taxonomic synonym: *Fusarium lolii*) is able to produce a number of substances that can cause adverse health effects in humans including mycotoxins. The production of mycotoxins has resulted in toxicosis in humans after consumption of rice contaminated with *Fusarium* (Wang *et al*., 1993).

A published review of mycotoxins (Sweeney and Dobson, 1999) outlined the biosynthesis of trichothecene mycotoxins in *Fusaria*. Seven genes from *F. sporotrichioides* have been characterised, however none of these genes encode a lipase enzyme. The applicant conducted a Medline literature search, which did not reveal a connection between lipases or phospholipases in the production of toxins in *Fusaria*. Furthermore, a lipase originating from another species, *F. oxysporum* is commercially available for the food market[12].

Fusarium species are known to cause local infections in humans, mainly in skin, nails and eyes, but rarely cause deep tissue infections. Systemic infections in immunocompromised patients have been reported with *F. solani*, *F. verticillioides*, *F. oxysporum*, *F. moniliforme*, *F. antophylum*, *F. proliferatum* and *F. chlamydosporum*. However, systemic infection has not been reported with *F. heterosporum*, the source of the lipase gene used for transformation of the production organism.

2.2 Safety of the host organism

The safety of the production organism is an important consideration in the safety assessment for enzymes used as processing aids. The primary issue is the potential toxicity of the production organism itself, that is, the possible synthesis of toxins by the production strain and the potential for the carryover of these into the enzyme preparation (Pariza and Johnson, 2001).

[12] Standard 1.3.3 Processing Aids, Clause 17 – Permitted Enzymes of Microbial Origin, Australia New Zealand Food Standards Code; US FDA, GRAS Notice No. GRN 000075

H. polymorpha is a strain of yeast belonging to the ascomycetes, with a two-phase life cycle entailing reproduction by budding, and by creation of ascospores. The recombinant micro-organism however, does not show this ability. Alternative names for *H. polymorpha* include *Pichia angusta*, *H. angusta* and *Torulopsis methanothermos*. The latter name reflects the ability of *H. polymorpha* to grow on methanol as the only carbon source.

The entire genome of *H. polymorpha* has been characterised. The genome of the RB 11 strain is organised as 6 chromosomes ranging in size from 0.9 to 2.2 megabases (Mb). There is no extra chromosomal DNA, nor dormant genes. It created interest as a production organism for heterologous proteins because of the presence of very strong promoters and the ability to secrete large amounts of protein. Like other fungal eukaryotes such as *Saccharomyces cerevisiae*, *H. polymorpha* is capable of glycosylating proteins.

The organism does not contain pyrogens, pathogens or viral inclusions. A study by Holzschu *et al*. in 1979 showed that *H. polymorpha* is neither fatal nor infective in mice. In the literature, there is one example of human infection with *H. polymorpha* in a patient with existing debilitating disease. Fungal infections in immunocompromised patients are relatively common, and in this case the infection was treated with one of the polyene antimicrobials, Amphotericin B. There are numerous examples in the literature of another species, *H. anomala*, being infectious to humans with a weakened immune system. The toxin from *H. anomala* induces acute damage in the small intestine of rats.

A recombinant strain of *H. polymorpha* RB11 has been developed for production of fungal phytase for feed use and for the enzyme hexose oxidase (ADI 'not specified', JECFA). In Australia/New Zealand, hexose oxidase produced by recombinant *H. polymorpha* has been evaluated and its use in food is permitted in the Code[13]. Recombinant strains of *H. polymorpha* are also used for the commercial production of hepatitis B vaccines. Several other proteins/enzymes are produced in recombinant *H. polymorpha* strains, and some are registered for use in various overseas countries. The literature therefore indicates that while other species of *Hansenula* and *Pichia* can be pathogenic, *H. polymorpha* is described as a safe production organism.

3. Manufacturing process

The manufacture of the enzyme preparation is a three-part process consisting of (i) fermentation of the production organism; (ii) recovery of the enzyme fraction from the cell mass and multiple filtration steps to concentrate the enzyme; and (iii) formulation/drying which is the preparation of a stable food-grade enzyme product. During the processing of the enzyme, preservatives may be added as processing aids. These could include sulphite, sodium benzoate, potassium sorbate and methyl- and propyl-p-hydroxybenzoate or their sodium salts.

4. Purity of enzyme preparation and proposed specifications

Historically, enzymes used in food processing have been found to be non-toxic, and the main toxicological consideration is in relation to possible contaminants. The production organism in this case is considered to be non-toxic and non-pathogenic. The specifications to which the preparation conforms are shown in Table 1.

[13] Standard 1.3.3 Processing Aids, Clause 17 – Permitted Enzymes of Microbial Origin, Australia New Zealand Food Standards Code.

Table 1: Complete specification of KLM1 sourced from *Hansenula polymorpha*

Criteria	Specification
Triacylglycerol acylhydrolase activity (TIPU/g)	1500
Total viable count (cfu/g)	$< 5 \times 10^4$
Total coliforms (cfu/g)	< 30
Enteropathogenic *E. coli*/25 g	negative by test
Salmonella sp./25 g	negative by test
Antibiotic activity	negative by test
Heavy Metals	< 40 ppm
Arsenic	< 3 ppm
Lead	< 5 ppm

The activity of KLM1 is defined in TIPU (Titratable Phospholipase Units); 1 TIPU is defined as the amount of enzyme that liberates 1 μmole free fatty acid per minute under the assay conditions. The assay is based on the enzyme's ability to hydrolyse lecithin to free fatty acids.

The lipase enzyme produced by *H. polymorpha* is approximately 30 kDa on SDS-PAGE. This corresponds well with the average molecular weight of 29 kDa determined by MALDI-TOF. The protein consists of 279 known amino acid residues.

KLM1 lipase from the production organism, *H. polymorpha*, complies with the relevant international Standards for Enzyme Preparations (JECFA, 2002; Food Chemicals Codex, 2004).

4. Nature of the genetic modification

The gene derived from *F. heterosporum* (CBS 782.83) was resynthesised in the laboratory with codon usage optimised for expression in yeast. The synthetic gene encodes the same amino acid sequence as the native gene from *F. heterosporum.*

Plasmid pB14-CBSsynt was used as vector for the insertion of the synthetic lipase gene into the host organism under the regulation of a promoter (FMD1), transcription terminator (MOX) and other genetic elements (HARS1) from *H. polymorpha.* There were no antibiotic resistance marker genes present in the vector.

The strain of *H. polymorpha* producing KLM1 was constructed by electroporation of the uracil auxotrophic strain RB11 to create the strain designated as B14-CBSsynt. Southern blot analyses determined that the transformed strain contains approximately 80 copies of the plasmid pB14-CBSsynt integrated into the chromosomal DNA.

Genetic stability

The approximate number of lipase genes from 3 different fermentations (grown for different number of generations) was determined by Southern blot analysis. Within the limitations of this analysis, the number of inserted genes was constant and therefore the genes are considered to be stably integrated into the host genome.

5. Evaluation of the safety studies

Four toxicological studies were submitted in support of this application, including the following:

1. Acute oral toxicity study in rats;
2. 90-day sub-chronic oral toxicity study in rats;
3. Bacterial Reverse Mutation Assay (Ames test); and
4. *In vitro* mammalian chromosome aberration test.

5.1 Potential toxicity and allergenicity of KLM1 lipase

The amino acid sequence of the lipase from *F. heterosporum* was compared to the sequences of known toxins and allergens to assess if there was any significant sequence homology.

No significant homology to any toxin sequence was found. No matches greater than 5 contiguous residues were found between known allergens and the lipase. It has been reported that an immunologically significant sequence similarity requires a match of at least 8 contiguous identical residues.

These data demonstrate that KLM1 lipase is unlikely to share structurally or immunologically relevant sequence similarities with known protein toxins or allergens.

5.2 Acute toxicity

Study details:
Acute Oral Toxicity of Lipase in the Rat, SafetyCity Ltd, Finland. Study No. SC 420302-04040. Director: Mari Madetoja; Date: 11 March, 2005.

Test material	Lipase KLM1
Vehicle material	sterile water
Test species	Sprague Dawley rats: Hsd:SD:Tu (1 animal/dose for the preliminary study, 4 animals/dose for the main study)
Dose	1.33 g/kg bw for both studies
GLP/guidelines	OECD (1997, 1998)/OECD guidelines (2000, 2001)

In the main study, a group of 4 female rats received a single dose of 1.33 g/kg bw of KLM1 lipase (equivalent to 82,000 U/kg bw). The selected dose derived from the preliminary study, which was based on information provided by the sponsor. Bodyweights were measured prior to dosing, at day 7, and before necropsy. Animals were observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, and daily thereafter for 14 days. Clinical signs were generally observed and recorded twice daily. Observations as a minimum included changes in:

- the skin and fur
- eyes and mucous membranes
- respiration/ respiratory organs
- circulation
- autonomic and central nervous system
- somatomotor activity
- behaviour

At termination, animals were weighed and gross necropsy was performed on all animals and macroscopic findings were recorded.

No treatment related signs were recorded on clinical examinations, and all animals survived in good condition throughout the course of the study. Body weight gains were normal in all animals during the observation period. In general, no macroscopic findings were noted at necropsy. In the abdominal cavity, hyperaemia in the ileum mucous was observed in one animal however the cause could not be determined. There were no signs of toxicity observed in the thoracic cavity. Under the conditions of this study, oral administration of KLM1 lipase at a dose of 1.33 g/kg bw was considered to produce no treatment-related effects.

5.3 Sub-chronic toxicity

Study details:
Subchronic Oral Toxicity Study – 90 day study in the Rat, SafetyCity Ltd, Finland. Study No. SC 240325-04039. Director: Mari Madetoja, Date: 21 June, 2005.

Test material	Lipase enzyme KLM1 (5,800 TIPU/ml, protein content 0.4%)
Control and vehicle material	Sterile water
Test Species	Sprague Dawley rats (4 groups of 10 males and 10 females)
Dose	Control 0 Low 5,800 U/kg bw/day Intermediate 17,400 U/kg bw/day High 58,000 U/kg bw/day by gavage
GLP	OECD (1997)
Guidelines	OECD guidelines 408 (1998)

Study conduct

Four groups of rats (10/sex/group) were administered lipase KLM1 by gavage at doses of 0, 5800, 17400 and 58000 U/kg bw per day for 91 days. Groups were designated group 1 (control), 2 (low dose), 3 (medium dose) and 4 (high dose).

The general well being of the animals was checked twice daily (once a day on the weekends). Clinical signs were observed daily at the same time as dosing and any abnormalities recorded. Detailed weekly observations included at least changes in:

- the skin and fur
- the eyes and mucous membranes
- respiration/ respiratory organs
- circulation
- autonomic and central nervous system
- somatomotor activity
- behaviour
- sensory reactivity
- assessment of grip strength

The animals were weighed individually before the experiment, on the first day of dosing, once a week during the course of the study and at necropsy. Animals in the control and high dose groups were subjected to ophthalmological examination at the beginning and end of the study. Food and water consumption was measured at intervals throughout the study. At the termination of the study, full necropsy was performed with organs weighed and macroscopic signs recorded. Details of the haematology, clinical chemistry, urinalysis and tissue sampling carried out in the study are presented in Appendix A. In addition, all organs listed were processed, embedded in paraffin, cut and stained for histopathological examination.

Results

All animals survived in good condition for the duration of the study.

No treatment-related clinical signs were noted. The ophthalmological examinations did not reveal any abnormal changes in the eyes. No treatment-related effects were observed in body weights or body weight gain, and there were no differences between test and control animals. There were statistically significant differences between the test and control groups in food and water consumption.

There were no treatment-related differences in organ weight between the control and any of the three test groups, including notably for liver weights. There were no differences between control and test groups in urinalysis.

A number of macroscopic observations were recorded. Enlarged lymph nodes occurred in 3 animals in G1 (controls), 1 animal in G2 (low dose), 4 animals in G3 (intermediate dose) and 4 animals in G4 (high dose). The enlargements were found in all groups in the study and were attributed to benign hyperplasia and not considered to be treatment-related. Similarly, hyperaemia in the small intestine occurred in a small number of animals across all groups. A small erosion in the stomach mucus was observed in one animal from each of G1 (control) and G3 (intermediate dose). The testicles were degenerated in one G4 animal. All reported findings were considered to be within the background incidence of findings reported in rats of this age and strain.

Examination of specified tissues from G1 (controls) and G4 (high dose) revealed a range of histological findings, which are summarised in Table 2. The changes in the adrenal glands (unilateral focal adrenocortical hypertrophy – grade 1) were more prevalent in the control animals. Unilateral or bilateral cortical cysts were a common finding both in control and treated animals. All lesions of the adrenals were considered to be coincidental.

There were sporadic changes of minimal to mild severity noted in the kidneys of both male and female rats. These changes were however more prevalent in control animals and were not considered to be biologically significant.

Overall, the histological findings were minor, not considered to be of functional significance and were not indicative of a chemically-induced pathology.

Table 2: Summary of the number of histological findings in different groups by organs

Organ	Number of histological findings in the group			
	G1 (controls)		G4 (high dose)	
	males	females	males	females
Liver	2	1	0	0
Kidneys	8	5	4	4
Lungs	2	1	1	2
Adrenals	7	6	10	5
Other organs*	3	0	4	2

* stomach, testes, skin, bladder

The results of the clinical chemistry analyses indicated a statistically significant increase ($p<0.05$) in the serum bilirubin level in treatment groups 3 and 4, compared to the control group (G1). The data indicate that the increase was dose-related, and occurred in both male and female animals. According to the study director, all observed values were within historical control values (data not supplied). Based on the clinical chemistry results, the target organ would be the liver, however there were no toxicological findings or observations indicating a treatment-related effect.

Conclusion

There were no findings in this study to indicate treatment-related systemic toxicity and target organs were not identified. Based on these results, the no-observed-adverse-effect-level (NOAEL) for KLM1 lipase is 58,000 U/kg bw per day, the highest dose tested in this study.

5.4 Genotoxicity studies

Reverse mutation test in bacteria

Study details:
KLM1 Bacterial Reverse Mutation Test, BioTest Ltd, Czech Republic. Study ID No. 032/04/L. Study Director: Jiri Marhan.

The bacterial reverse mutation test is a short-term test used to identify a potential to cause point mutations or frameshift mutations in genetic material.

Test article

KLM1 lipase (IUB 3.1.1.3), batch # TOX1, freeze dried powder, lipase activity 62,000 TIPU/g
Purity: dry matter content 99.1%

Study design

Lipase KLM1 was examined for mutagenic activity in four (histidine) auxotrophic strains of *Salmonella typhimurium* (TA97, TA98, TA100, TA1535) and a (tryptophan auxotrophic) strain of *Escherichia coli* (WP2uvrA).

Experiments were performed with and without *in vitro* metabolic activation. KLM1 was dissolved in water immediately before use and doses of 0.01, 0.1, 0.5, 2 and 5 mg were applied per plate. The method used was according to OECD Guidelines No. 471.

The procedure was based on the plate-incorporation method according to the standard procedure of Maron and Ames (1983), Green (1984) and Gatehouse *et al*. (1994). A preliminary test was carried out to find a suitable dose range using *S. typhimurium* strain TA100, with and without S9 metabolic activation. The five doses used in the main assays were deduced from the levels of solubility and toxicity. The mutagenicity plate test was conducted twice in two independent assays using four *S. typhimurium* strains and *E. coli* WP2uvrA. The five doses of the test material were used on each of the 5 bacterial strains, with 5 mg/plate as the highest dose level, both with and without metabolic activation. Each test point was carried out in triplicate. The sensitivity of the individual bacterial strains was confirmed by significant increases in the number of revertant colonies induced by diagnostic mutagens (sodium azide, N-ethyl-N'-nitro-N-nitrosoguanidine, 2-nitrofluorene, 2-aminoanthracene, cyclophosphamide). Negative controls consisted of 100 µl vehicle applied to each plate.

Test	*Test material*	*Concentration*	*Test object*	*Result*
Reverse mutation (*In vitro*)	lipase KLM1	**test 1** : 0, 10, 100, 500, 1000, 2500 and 5000 µg per plate **test 2**: 0, 10, 100, 500, 1000, 2500 and 5000 µg per plate	*Salmonella typhimurium* TA97, TA98, TA100, TA1535 and *Escherichia coli* WP2uvrA	-ve

Results and conclusion

No dose-related increases in mutation frequency were observed in the strains tested. It was concluded that lipase KLM1 produced by *H. polymorpha* did not exhibit mutagenic activity under the conditions of the test.

***In vitro* mammalian chromosome aberration test**

Study details:
KLM1 *In vitro* Mammalian Chromosome Aberration Test, BioTest Ltd, Czech Republic. Study ID No. 033/04/L. Study Director: Jiri Marhan.

The *in vitro* mammalian chromosome aberration test is a short-term test used to identify structural chromosome aberrations in cultured mammalian cells. Detected structural aberration may be chromosome- or chromatid-type.

Test article

Lipase KLM1 (batch no. TOX1), freeze dried powder, lipase activity 62000 TIPU/g.

Study design

Lipase KLM1 was tested in an *in vitro* cytogenetics assay using human peripheral blood lymphocytes, with and without *in vitro* metabolic activation (S9), in two separate experiments. The study was conducted in accordance with OECD guideline 473 (1997). The method allows detection, qualitative and quantitative analysis of chromosomal abnormalities (structural and numerical types) in human peripheral lymphocytes.

In the preliminary dose-finding test, cell cultures were exposed to the test substance for 4 hours and 48 hours, both with and without metabolic activation, at 5 dose levels – 1, 2, 3, 4 and 5 mg of dry test material per ml of culture. The doses used for the main study were selected by determining 3 dose levels showing toxicity. The degree of toxicity was evaluated microscopically according to the following criteria:

- strong toxicity – no cell proliferation, no mitosis
- moderate toxicity – limited cell growth and proliferation, low mitotic index (%MI)
- slight toxicity – no or slight visible proliferative changes, %MI lower than half negative control value.

Strong or moderate toxic effects were observed at a dose of 4 and 5 mg/ml of test substance, and slight toxicity was observed as a dose of 3 mg/ml with and without metabolic activation.

In the mutagenicity experiment, cell cultures were exposed to three dose concentrations of the test substance (0.5, 1 and 2 mg/ml) both with and without metabolic activation. At predetermined intervals after exposure (4h and 48h), the cells were treated with colchicine to arrest cell division in metaphase. Phytohaemagglutinen (lectin) was used as a lymphocyte mitogen. Negative and positive controls were included in each test. All cultures for the 3 different doses were performed in duplicate. A positive result is recorded if the test substance increases the average % frequency of aberrant cells to more than twice that of the negative control. The % frequency of aberrant cells did not increase with any dose greater than the control value, showing no concentration-related effects, even at the 48 hour sampling time. These negative results were considered by the authors to be biologically relevant and reproducible.

Results and conclusion

Treatment with KLM1 lipase did not produce increases in the frequency of aberrant chromosomes at any concentration tested when compared to control values, either in the presence or absence of *in vitro* S9 metabolic activation.

6. Overall Conclusion

The conclusions from the safety assessment of KLM1 lipase from *H. polymorpha* are:

- the production organism has a history of safe use as a production strain for food-grade enzyme preparations and has been shown not to produce toxic metabolites;
- the recombinant DNA in the production organism is considered to be stable and poses no safety concern;
- the enzyme preparation complies with international specifications;

- there was no evidence of toxicity in the sub-acute toxicity study or in the sub-chronic toxicity study in rats;
- in the sub-chronic study in rats, the no-observed-adverse-effect-level (NOAEL) was 58,000 U/kg bw per day which was the highest dose tested; and
- the enzyme preparation produced no evidence of genotoxic potential in *in vitro* assays in bacteria and in human peripheral blood lymphocytes.

From the available information, it is concluded that the use of KLM1 lipase as a processing aid in food would pose no public health and safety risk.

References:

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Sweeney, M.J. and Dobson, A.D.W. (1999) Molecular Biology of Mycotoxin Biosynthesis. Fems Microbiol. Letters 175, 149-163.

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APPENDIX A

Analyses performed in 90-day oral toxicity study in rats of KLM1

Haematology parameters	
Haemoglobin	Red blood cell count
Haematocrit	Mean corpuscular volume of RBC
Mean cell haemoglobin	Mean corpuscular haemoglobin conc.
Total leucocyte count	Prothrombin time
Platelet count	Activated Partial Thromboplastin Time
Clinical chemistry	
Alanine aminotransferase, serum	Sodium, serum
Aspartate aminotransferase, serum	Potassium, serum
Alkaline phosphatase, serum	Calcium, serum
Protein, serum	Phosphorus, serum
Total Cholesterol	Albumin, serum
Triglycerides, serum	Bilirubin, serum
Urea, serum	Glucose, serum
Creatinine, serum	
Urinalysis	
Volume	Protein
pH	Glucose
Specific gravity	Blood
Organs weighed	
Adrenals	Uterus
Kidneys	Ovaries
Liver	Spleen
Testes	Thymus
Epididymes	Heart
	Brain
Pathology	
All tissues with macroscopic changes	Lymph nodes
Heart	Oesophagus
Lungs	Pancreas
Pituitary	Prostate
Salivary glands	Peripheral nerve
Seminal vesicles	Skeletal muscle
Liver	Spinal cord
Brain	Sternum (including bone marrow)
Stomach	Skin with mammary gland
Kidneys	Spleen
Adrenals	Thymus
Small intestine (2 samples, incl. Peyers patches)	Thyroids & parathyroids
Trachea	Urinary bladder
Large intestine	Testicles
Ovaries	Epididymes
Aorta	Uterus
Bone (distal femur)	

Attachment 3

Food Technology Report

A569 – Lipase from *Hansenula polymorpha* as a processing aid (enzyme)

Introduction

FSANZ received an Application from Danisco Australia Pty Ltd to amend the *Australia New Zealand Food Standards Code* (the Code) to approve an enzyme lipase triacylglycerol sourced from *H. polymorpha* containing the gene coding for lipase triacylglycerol isolated from *F. heterosporum* as a processing aid.

Lipase triacylglycerol

In the Table to clause 17 – Permitted enzymes of microbial origin of Standard 1.3.3 of the Code the name of this enzyme is lipase triacylglycerol. Its common name is lipase, with other names including triacylglycerol lipase (being the name conferred to this enzyme by the International Union of Biochemists and Molecular Biochemists (IUBMB)), triglyceride lipase and tributyrase. This enzyme is already approved in the Code with a number of other sources, not including *H. polymorpha* containing the gene coding for lipase triacylglycerol isolated from *F. heterosporum*.

Lipase triacylglycerol has the Enzyme Commission (EC) number of [3.1.1.3] and a Chemical Abstracts System (CAS) number of 9001-62-1.

There is another lipase listed in Table to clause 17 of the Code, but this is called lipase, monoacylglycerol which is a different enzyme with an EC number of [3.1.1.23].

Lipase (EC [3.1.1.3]) is also listed in Table to clause 15 – Permitted enzymes of animal origin of the Code. This enzyme is sourced from bovine stomach; salivary glands or forestomach of calf, kid or lamb; porcine or bovine pancreas.

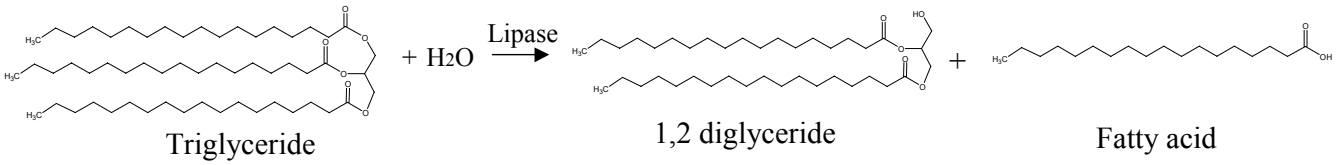
The enzyme for this Application is produced using recombinant DNA techniques from a microbial source (the host yeast *H. polymorpha* containing the gene coding for lipase triacylglycerol isolated from *F. heterosporum*) rather than an animal source.

The enzyme preparation is an off-white to brown powder with pH stability between 5 and 7 and optimum activity at pH of approximately 8. The optimum temperature of use is approximately 40ºC. It is thermally stable below 37ºC in an aqueous solution, and not stable above 45ºC.

Lipases are enzymes that catalyse the cleavage of triglycerides, phospholipids and galactolipids to fatty acids. The enzyme is characterised by its ability to catalyse the reaction:

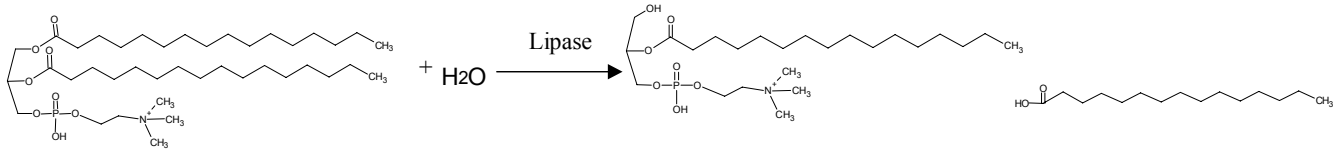
$$\text{Triacylglycerol} + H_2O \rightarrow \text{Diacylglycerol} + \text{a fatty acid anion}$$

The following schematics of how the enzyme reacts with triglycerides, phospholipids and galactolipids has been taken from the Application.



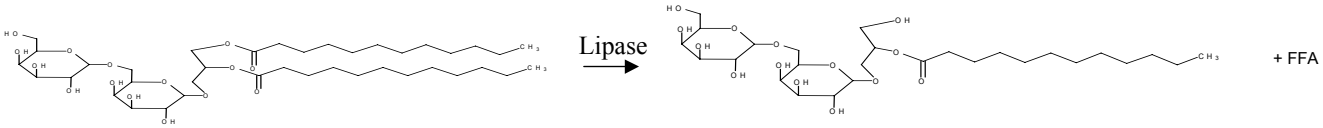
Triglyceride 1,2 diglyceride Fatty acid

The enzyme also has activity towards sn-1 ester bonds in other lipid components including diacyl-phospholipids, e.g.:



Phosphatidylcholine Lyso-phosphatidylcholine Fatty acid

and diacyl-galactolipids, e.g.:



Digalactosyl diglyceride Digalactosyl monoglyceride

Technological justification

The Applicant proposes that the enzyme would be used in a number of food industries, including bread manufacture, general baking, in the pasta and noodle production, in the use of egg yolk and whole eggs in food and for degumming of oils.

The major food application would be expected to be in break making. Wheat flour used for bread manufacture contains approximately 2% lipids (various triglycerides, diglycerides, phospholipids and galactolipids) which are modified by the enzyme. The cleaved reaction products are more polar than the initial lipids and help to produce bread with improved volume and homogeneous crumb structure. The Applicant states that these more polar lipids improve dough stability and handling properties.

The analogous situation that occurs for bread manufacture also occurs for noodle and pasta manufacture. The use of the enzyme on the wheat flour used to produce noodles and pasta modifies the wheat lipids to more polar products which improves the quality of the final noodle or pasta product and lower cooking losses.

Approximately 30% of the egg yolk lipid is phospholipids which is reacted by the enzyme to produce a product that has improved emulsifying properties. The enzyme modified egg yolk can be used to produce mayonnaise with less phase separation that can occur when mayonnaise is pasteurised at high temperatures.

Edible oils such as soybean oil contains some phospholipids which need to be removed during the refining process to improve the oil quality and taste. Treating the oil with the enzyme during processing hydrolyses the phospholipids to the more polar lyso-phospholipids which can be removed from the refined oil using water extraction.

The Table below has been taken from the Application to indicate the levels of the enzyme used in different food types.

Food	**Use level units/kg food**
Bread	100-4000
Eggs via bakery products	250-1000
Eggs via mayonnaise	5000-20000
Oils	200-2000

The Applicant tested the activities of the lipase enzyme against a number of other substrates rather than various lipids to assess any extra activity. The results were negative for alpha-amylase, endo-xylanase, protease, glucose oxidase and beta-glucanase, indicating that the enzyme has reasonable purity with no unintended activity to other substrates.

Production of the enzyme

The lipase triacylglycerol enzyme preparation is produced by submerged fermentation using a selected strain of the yeast *Hansenula polymorpha* that has the gene coding for lipase triacylglycerol isolated from *Fusarium heterosporum* inserted by recombinant DNA techniques. After fermentation is completed the biomass is removed by centrifugation and filtration. The supernatant fermentation broth which contains the enzyme is filtered and then concentrated by ultra-filtration. The ultra-filtrate is then sterile filtered and finally spray dried or granulated onto a food grade carrier such as wheat starch. The manufacturing process is that commonly used to produce enzymes from microbial sources.

Specification

The Application states that the enzyme preparations meet the international specifications for enzymes contained in the Food Chemical Codex (5th Edition, 2004), and the Joint FAO/WHO Expert Committee on Food Additives (JECFA), in the Compendium of Food Additives Specifications, Vol 1 Annex 1, FAO 1992 (Addendum 9, 2001). The specification below is taken from the Applicant's enzyme specification supplied.

Criteria	**Specification (meets or exceeds JECFA)**
Heavy Metals as Pb	not more than 40 ppm
Arsenic	not more than 3 ppm
Lead	not more than 5 ppm
Total viable count (cfu/g)	not more than 50,000
Total coliforms (cfu/g)	not more than 30
Antibiotic activity	negative by test
Salmonella (/25 g)	negative by test
Escherichia coli (/25 g)	negative by test

Conclusions

The use of the enzyme lipase triacylglycerol sourced from the host yeast *Hansenula polymorpha* containing the gene coding for lipase triacylglycerol isolated from the mould *Fusarium heterosporum* as a processing aid is technologically justified to improve food manufacturing in a number of industries. These including bread making, general baking, noodle and pasta manufacture, the use of eggs and edible oil industries.

References

References used for specific background on the enzyme

Enzyme Nomenclature, International Union of Biochemists and Molecular Biochemists (IUBMB) Academic Press, Inc, 1992.
and more updated reference also found at www.chem.qmul.ac.uk/iumbm/enzyme/

Expert Protein Analysis System (ExPAS)
http://us.expasy.org/cgi-bin/enzymes-search-ec

University College London, Enzyme Structure Database
www.biochem.ucl.ac.uk/bsm/enzymes/

General references on lipases

Pandey, A.; Benjamin, S.; Soccol, C.R.; Nigam, P.; Krieger, N. and Soccol, V.T. (1999) The realm of microbial lipases in biotechnology, *Biotechnol. Appl. Biochem.*, **29**, 119-131.

R.K. Saxena, P.K. Ghosh, R. Gupta, W.S. Davidson, S. Bradoo and R. Gulati., Microbial lipases: Potential biocatalysts for the future industry, http://www.ias.ac.in/currsci/jul10/articles18.htm,

Specifications

Food Chemicals Codex, (2004). National Academy of Sciences, Food and Nutrition Board, Committee on Food Chemical Codex, 5th Edition, National Academy Press, Washington DC.

Joint FAO/WHO Expert Committee on Food Additives (JECFA) (2001), Compendium of Food Additives Specifications, General specifications and considerations for enzyme preparations used in food processing. FAO Food and Nutrition Paper 52, Add. 9, pp 37-39.

Attachment 4

Summary of Submissions

Round One

Submitter Organisation	Name
Food Technology Association Vic	David Gill
Private	Margaret and Bob Aylward
New Zealand Food Safety Authority	Carole Inkster
Private	Ivan Jeray
Department of Health, South Australia	Joanne Cammans
Australian Food and Grocery Council	Kim Leighton
New South Wales Food Authority	Kelly Boulton

Submitter	Position	Comments
Food Technology Association Vic	Agrees, supports option 2	Supports the Application
Margaret and Bob Aylward	Do not support	Support amending labelling regulations to ensure that a genetically modified strain of the micro-organism was used to provide accurate information to the consumer.
New Zealand Food Safety Authority	No position stated	Submission pointed out an error, where the incorrect donor organism when mentioned, i.e. F. venenatum instead of F. heterosporum, in section 5.2 of the report.
Department of Health, South Australia	Cautious support	Conditional support awaiting the results of the safety assessment.
Ivan Jeray	Do not support	Does not support approval of any food that has been genetically modified or contains genetically modified ingredients.
Australian Food and Grocery Council	Agrees, supports option 2	Support for the use of processing aids for the food industry use, provided they are safe and fulfil a technological function. Notes that previous approvals of the host yeast demonstrated that there are no public health and safety concerns with using this micro-organism, and notes FSANZ's conclusion that there are no public health and safety concerns. Noted that there is a technological justification for the use of the enzyme. Technological justification is not part of FSANZ's section 10 objectives. Believes that technological use is more an issue for industry than for FSANZ, but are sure that FSANZ will find its use is technologically justified. The safety of lipase *per se* is not required to be assessed for safety as it has previously been assessed as safe. But rather the safety focus should be on any issues arising from deriving the enzyme from the source micro-organism.
New South Wales Food Authority	Supports	No concerns at this stage and support further consideration of the Application.

Round Two

Submitter Organisation	Name
Private	Ivan Jeray
Food Technology Association Victoria	David Gill
New Zealand Food Safety Authority	Carole Inkster
Australian Food and Grocery Council	Kim Leighton
Department of Human Services Victoria	Victor Di Paola
Queensland Department of Health	Gary Bielby
Private	Paul Elwell-Sutton

Submitter	Position	Comments
Ivan Jeray	Do not support option 2	Does not support approval of any food that has been genetically modified or contains genetically modified ingredients.
Food Technology Association Victoria	Supports option 2	The committee accepted the approval of lipase derived from *Hansenula Polymorpha* containing the gene coding for lipase triacylglycerol from *F. heterosporum* as a Processing Aids
New Zealand Food Safety Authority	Supports option 2	NZFSA supports option 2 and suggest that the source organism be referred to as *Pichia angusta*.
Australian Food and Grocery Council	Supports option 2	Support for the use of processing aids for the food industry use, provided they are safe and fulfil a technological function. After FSANZ second round of public consultation AFGC supports the approval for the use of lipase derived from *Hansenula Polymorpha* as a Processing Aids without reservations.
Department of Human Services Victoria	Supports Option 2	The DHS Victoria accepts the option 2 to include lipase from *Hansenula Polymorpha* as Processing Aids, since it is technologically justified and does not raise any public health and safety concerns.
Queensland Department of Health	Supports Option 2	The Queensland DH accepts the option 2 to include lipase from *Hansenula Polymorpha* as Processing Aids
Private	Do not support option 2	Does not support approval of any food that has been genetically modified or contains genetically modified ingredients.