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Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document

Risk and technical assessment – Application A1301

Triacylglycerol lipase from GM *Komagataella phaffii* as a processing aid

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Executive summary

Danstar Ferment AG, an affiliate of Lallemand Inc. has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme triacylglycerol lipase, EC 3.1.1.3. Triacylglycerol lipase is produced from a genetically modified (GM) strain of *Komagataella phaffii* containing the lipase gene from *Fusarium oxysporum*.

The enzyme is intended to be used as a processing aid in the manufacture of bread and bakery products. The proposed use is technologically justified for use at levels consistent with GMP.

There are relevant identity and purity specifications for the enzyme in the Code and the applicant provided evidence that their enzyme meets these specifications.

K. phaffii (previously named *Pichia pastoris*) has a long history of safe use as a production microorganism of enzyme processing aids. The production organism is neither pathogenic nor toxigenic. Analysis of the modified production strain confirmed the presence and stability of the inserted DNA.

Sufficient information has been provided to assess the safety of the triacylglycerol lipase that is the subject of this application. While a history of safe use for this specific enzyme has not been established, the production organism itself has a long history of safe use and raises no issues regarding the presence of secondary metabolites of toxicological concern in the enzyme preparation. The enzyme itself is rapidly destroyed under conditions replicating those in the human stomach and duodenum, and no significant homology between the enzyme and any known toxins or allergens was identified.

Based on the safety assessment and considering the theoretical maximum daily intake (TMDI) (0.127 mg Total Organic Solids (TOS)/kg body weight (bw)/day), no public health and safety concerns were identified in the assessment of the triacylglycerol lipase produced by this GM *K. phaffii* under the proposed conditions.

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1 Introduction

Danstar Ferment AG, an affiliate of Lallemand Inc. (the applicant) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme triacylglycerol lipase, EC 3.1.1.3. This triacylglycerol lipase is produced from a genetically modified (GM) strain of *Komagataella phaffii* containing the lipase gene from *Fusarium oxysporum*. The specific name for the production strain used by the applicant is *K. phaffii* stain LALL-LI2.

The enzyme is intended to be used as a processing aid in the manufacture of bread and bakery products. The stated purpose is to improve dough structure and behaviour during baking, increase bread volume and improve crumb structure. The usage level is the minimum level required to achieve the desired effect, in accordance with the principles of Good Manufacturing Practice (GMP)[1].

1.1 Objectives of the assessment

The objectives of this risk and technical assessment were to:

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

[1] GMP is defined in the Standard 1.1.2—2 of the Code as follows: *with respect to the addition of substances used as food additives and substances used as processing aids to food, means the practice of:*
(a) limiting the amount of substance that is added to food to the lowest possible level necessary to accomplish its desired effect; and
(b) to the extent reasonably possible, reducing the amount of the substance or its derivatives that:
*(i) remains as a *component of the food as a result of its use in the manufacture, processing or packaging; and*
(ii) is not intended to accomplish any physical or other technical effect in the food itself

2 Food technology assessment

2.1 Identity of the enzyme

The applicant provided relevant information regarding the identity of the enzyme, and this has been verified using the IUBMB[2] enzyme nomenclature reference database (McDonald et al 2009).

Accepted IUBMB name:	Triacylglycerol lipase
Systematic name:	Triacylglycerol acylhydrolase
Other names/common names:	Lipase, triglyceride lipase, glycerol ester hydrolase, tributyrase, butyrinase, tributyrinase, tributyrin esterase, triglyceride hydrolase; triglyceridase; triacylglycerol ester hydrolase
IUBMB enzyme nomenclature:	EC 3.1.1.3
CAS number:	9001-62-1
Reaction:	Triacylglycerol lipase or lipase (EC 3.1.1.3) catalyses the hydrolysis of triglycerides ester bonds into diglycerides and subsequently into monoglycerides and glycerol, as well as free fatty acids

triacylglycerol + H_2O = diacylglycerol + a carboxylate

2.2 Manufacturing process

2.2.1 Production of the enzyme

Enzymes produced from microorganisms are typically produced by controlled fermentation followed by removal of the production microorganism, purification and concentration of the enzyme. Final standardisation with stabilisers, preservatives, carriers, diluents, and other approved food-grade additives and ingredients is carried out after the purification and concentration steps. The formulated enzymes are referred to as enzyme preparations, which, depending upon the application in food, may be a liquid, semi-liquid or dried product. Enzyme preparations may contain either one major active enzyme that catalyses a specific reaction during food processing or two or more active enzymes that catalyse different reactions (FAO/WHO 2020a).

All the raw materials and processing aids used in the manufacture of the enzyme preparation are food grade, acceptable for use and used in the manufacture of food enzymes and other food production processes.

2.2.2 Specifications for identity and purity

There are international general specifications for enzyme preparations used in the production of food. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its Compendium of Food Additive Specifications (FAO JECFA Monographs 26 (2021)), explicitly FAO/WHO (2006) and in the Food Chemicals Codex (FCC

[2] International Union of Biochemistry and Molecular Biology.

2022), referenced in subsection 3—2 of Schedule 3 of the Code. Enzymes used as a processing aid need to meet either of these specifications, or a relevant specification in section S3—3 of Schedule 3. In addition, under JECFA, enzyme preparations must meet the specifications criteria contained in the individual monographs. In the case of triacylglycerol lipase produced from a GM strain of *K. phaffii*, there is no individual monograph.[3]

Schedule 3 of the Code also includes specifications for arsenic and heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

The applicant provided the results of analysis of three different batches of their triacylglycerol lipase. Table 1 provides a comparison of the results of those analyses with international specifications established by JECFA and Food Chemicals Codex, as well as those in the Code (as applicable). Based on those results, the enzyme met all relevant specifications.

Table 1 *Analysis of manufacturer's final enzyme preparation (triacylglycerol lipase produced from a GM strain of Komagataella phaffii) compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes.*

Test parameters	*Danstar Ferment AG test results**	*Specifications*		
		JECFA	*Food Chemicals Codex*	*The Code - section S3—4*
Lead (mg/kg)	0.028, 0.047, 0.025	≤5	≤5	≤2
Arsenic (mg/kg)	≤0.02	-	-	≤1
Cadmium (mg/kg)	≤0.008	-	-	≤1
Mercury (mg/kg)	≤0.01	-	-	≤1
Coliforms (cfu/g)**	≤10	≤30	≤30	-
***Salmonella* (in 25 g)**	Not detected	Absent	Negative	-
***Escherichia coli* (in 25 g)**	Not detected	Absent	-	-
Antimicrobial activity***	Absent	Absent	-	-
Production Strain***	Absent			

*Where all three batch results are the same, only one is listed.
**cfu = colony forming units.
***Information provided in confidential appendix.

The specification for the enzyme preparation used by the manufacturer (as provided in section A.5 of the application) includes a test for the absence of the production strain. Refer to Section 3.4 below for the total organic solids (TOS) value. TOS encompasses the enzyme component and other organic material originating from the production organism and the manufacturing process, while excluding intentionally added formulation ingredients.

2.3 Technological purpose

Under the current application, triacylglycerol lipase is intended for use as a processing aid in the manufacture of bread and bakery products. The applicant requested use of the enzyme at GMP levels.

[3] For the functional use 'enzyme preparation', the JECFA database can be searched for individual monographs: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/

Different triacylglycerol lipases have different substrate specificities and can therefore be used for a range of product applications (Geritis et al 2014, Melis et al 2017).

As identified by the IUBMB (section 2.1, above), triacylglycerol lipase catalyses the hydrolysis of triglycerides ester bonds into diglycerides and subsequently into monoglycerides and glycerol, as well as free fatty acids. As explained in the application, in the production of bread and bakery products, the enzyme reacts with the triglycerides contained in wheat flour. Lipases can also act on phospholipids and galactolipids converting them to more efficient emulsifying structures like lysophospholipids and digalactosyl monoglycerides (Gerits et al. 2014). These lipase-generated emulsifiers can interact with key dough components and enhance the dough stability and dough development. Finished products have improved bread volume, crumb structure and shape.

The technological purpose as stated by the applicant of improving crumb structure and bread volume in bread and bakery products is consistent with the stated function of triacylglycerol lipase and is supported by general information in the literature (Geritis et al 2014, Melis et al 2017), and also by information provided by the applicant in the confidential appendix. The applicant states that the triacylglycerol lipase subject to this application demonstrates high productivity, therefore providing industry with an alternative option when using a lipase in bread and bakery processes.

The applicant provided information on the physical and chemical properties of their enzyme preparation. Table 2 summarises this information. The enzyme is heat-denatured at a temperature of 60°C. Therefore, the enzyme is inactivated during the baking process and would have no technological function in final bread and bakery products.

Table 2 *Triacylglycerol lipase enzyme preparation physical/chemical properties*

Physical/chemical properties of commercial enzyme preparation	
Enzyme activity[4]	> 10,000
Appearance	Dried powder
Temperature range	Optimum activity within range 30 - 37°C
Temperature stability	The enzyme is completely deactivated after 15 min at temperatures above 60°C
pH range and optimum	Optimum activity within range 5 - 7

2.4 Allergen considerations

According to the applicant, the yeast used in the fermentation media is utilised by the production strain during fermentation, and the yeast biomass and fermentation solids are removed during downstream processing. The application also states that the enzyme preparation is free from known allergens. In particular, the glucose used in the fermentation process is not sourced from wheat, and the maltodextrin used in the formulation as a carrier is sourced from corn syrup.

2.5 Food technology conclusion

FSANZ concludes that the use of this triacylglycerol lipase as a processing aid for use in

[4] The enzyme activity is expressed in Lallemand Baking Lipase Units/g (LBLU/g). One LBLU is defined as the enzyme quantity that produces 1 micromole of butyric acid per minute at 21°C and pH=7.

bread and bakery products is consistent with its typical function of catalysing the hydrolysis of triglyceride ester bonds and also acting on phospholipids and galactolipids, converting them to more efficient emulsifying structures. The use of triacylglycerol lipase improves crumb structure, bread volume and shape in bread and bakery products. FSANZ concludes that the evidence presented to support its proposed use provides adequate assurance that the use of the enzyme, in the quantity and form proposed to be used (which must be consistent with GMP), is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

Triacylglycerol lipase performs its technological purpose during the manufacture of bread and bakery products, during which it is inactivated by heat, and is not performing a technological purpose in the final food. It is therefore functioning as a processing aid for the purposes of the Code.

There are relevant identity and purity specifications for the enzyme in the Code, and the applicant provided evidence that the enzyme meets these specifications.

3 Safety assessment

The objectives of this safety assessment are to evaluate any potential public health and safety concerns that may arise from the use of this enzyme, produced by this microorganism, as a processing aid.

Some information relevant to this section is Confidential Commercial Information (CCI), so full details cannot be provided in this public report.

3.1 Source microorganisms

3.1.1 Host and production organism

During the past couple of decades *Komagataella phaffii* has undergone a change in its nomenclature, and the organism is still sometimes referred to by its previous name *Pichia pastoris*. The name change to *K. phaffii* was first proposed by Yamada et al (1995). Genomic characterization by Kurtzman CP, provided further evidence supporting the identity of *K. phaffii* strains (2005 and 2009). *K. phaffii* is a Biosafety Level 1, methylotrophic yeast that has been used in the manufacture of pharmaceutical compounds, animal feeds, food enzymes and proteins since the 1980s (Barone et al, 2023; Spohner et al 2015). *K. phaffii* strains are isolated from the exudates of trees. The strain of *K. phaffii* used by the applicant was originally isolated from black oak trees in California, USA (Bernauer et al, 2020).

K. phaffii has been granted qualified presumption of safety (QPS) status by the European Food Safety Authority for the production of enzymes to be added to food (EFSA 2024). The restriction of QPS status to the production of enzymes is a reflection of the body of knowledge relating predominantly to use of *K. phaffii* in protein expression systems and as a model organism (EFSA 2018). To maintain QPS status, there must be an absence of viable cells of the production organism in the final product (EFSA 2024). *K. phaffii* has a long history of safe use for the production of pharmaceuticals and industrial chemicals, including a number of enzyme processing aids approved by EFSA, US FDA and FSANZ (Spohner et al. 2015).

The production strain, LALL-LI2, was created by inserting the lipase gene from *Fusarium oxysporum* into the wild type *K. phaffii* strain NRRL Y-11430 (ATCC 76273) (see Section 3.2 for further details). The NRRL Y-11430 strain is a non-toxigenic and non-pathogenic strain that has a history of safe use in manufacturing proteins for use in food, feed and

pharmaceuticals (Lynch et al, 2023; Reyes et al 2021).

Whole genome sequencing analysis (CCI data) was provided to FSANZ by the applicant. This information confirmed the identity of the production organism as *K. phaffii*. The applicant also demonstrated the absence of the production organism in the final enzyme product with data from three representative product batches. The production organism was also shown to be absent of antimicrobial resistance and mycotoxin genes.

The microbiological risk assessment undertaken by FSANZ did not identify any public health and safety concerns associated with the use of *K. phaffii* as a production organism for triacylglycerol lipase.

3.1.2 Gene donor organism

FSANZ has assessed previous processing aids that utilised *F. oxysporum* enzymes, including triacylglycerol lipase. The donor gene sequence was artificially synthesised from a known Genbank sequence. A comparison of the lipase gene sequence provided by the applicant to the *F. oxysporum* genome confirmed the donor organism identity.

3.2 Characterisation of the genetic modification

3.2.1 Description of the DNA to be introduced and method of transformation

A synthetic version of the gene encoding the native triacylglycerol lipase enzyme from *F. oxysporum* was introduced into specific locations in the genome of the host *K. phaffii* by homologous recombination, using standard molecular biology techniques. The lipase gene was under the control of native *K. phaffii* promoters and terminators. Data provided by the applicant and analysed by FSANZ confirmed the identity of the triacylglycerol lipase enzyme.

3.2.2 Characterisation of inserted DNA

Whole genome sequencing data provided by the applicant confirmed the presence of the inserted DNA at the intended locations in the genome of the production strain. The sequencing results also confirmed the absence of antibiotic resistance genes or any other unintended insertions in the production strain.

3.2.3 Genetic stability of the inserted gene

The assessment confirmed that the inserted gene is integrated into the genome of the production strain and does not have the ability to replicate autonomously. The inserted gene is therefore considered to be genetically stable.

To provide further evidence of the stability of the inserted lipase gene, the applicant provided PCR genotyping data on DNA isolated before and after large-scale fermentation of the production strain, which confirmed that the inserted DNA is stable over multiple generations.

3.3 Safety of the triacylglycerol lipase enzyme

3.3.1 History of safe use of the enzyme

The specific triacylglycerol lipase that is the subject of this application has not yet been assessed by any other regulatory agency, and no information on history of safe use is available.

The same enzyme from the same donor organism, *F. oxysporum,* but produced by a different

production organism (a strain of *Saccharomyces cerevisiae*) has been approved by EFSA and by Health Canada.

Closely related lipase enzymes from the donor organism *Fusarium oxysporum* but expressed by different production organisms have been approved for use by some regulatory agencies. FSANZ has approved such a lipase expressed by a strain of *Aspergillus oryzae* and another expressed by a strain of *Trichoderma reesei.* The latter enzyme has also been approved by Health Canada and by EFSA.

Triacylglycerol lipases as a functional group have a long history of safe use in food production and numerous triacylglycerol lipases have been approved by FSANZ and included in Schedule 18 of the Code.

3.3.2 Bioinformatics concerning homology with known toxins

Results of a recent (2023) bioinformatics search for similarity of the amino acid sequence of triacylglycerol lipase to those of known toxins were submitted as part of the application. A custom FASTA database of known toxins was created by searching the UniProtKB database (https://www.uniprot.org/) with the terms “keyword:toxin”. The amino acid sequence of the triacylglycerol lipase that is the subject of this application was queried against the custom toxin database using the BLAST function in the software Geneious Prime. There were two hits, both of which cover less than 30% of the lipase query sequence (only 82 residues and 93 residues, respectively) and both of which share less than 40% sequence identity with the query sequence across that interval. The E-values[5] were greater than 0.01 in both cases. A meaningful degree of homology is inferred only if the E-value is less than 0.01 (Pearson 2000).

3.3.3 Stability of the enzyme in simulated gastrointestinal systems

The triacylglycerol lipase from *F. oxysporum*, produced by a different production organism (a strain of *S. cerevisiae*) is the same protein as the enzyme that is the subject of this application, and was used to assess the stability of the enzyme in a simulated gastrointestinal digestion system. A detailed confidential report was provided to FSANZ for assessment.

The assay was conducted in triplicate, under fed conditions, using a Simulator of the Human Microbial System (SHIME®). The test article was introduced into the system at 350 mg, equivalent to a human dose of 5 mg powdered enzyme/kg bw, or 0.625 mg TOS/kg bw for a person weighing 70 kg. For the gastric phase, stomach conditions were simulated with stirring for 2 h at 37°C, while the pH in the reactor was decreased from 5.5 to 2.0, in the presence of pepsin, phosphatidylcholine, nutritional medium and salts. The contents of the reactor were sampled at 0 and 120 min. After 2 h the conditions were altered to replicate those of the small intestine by increasing the pH from 2.0 to 5.5 within 5 min, from 5.5 to 6.5 in the first hour and 6.5 to 7 in the second hour, followed by a third hour at a constant pH of 7.0. Pancreatic enzyme release was simulated by addition of trypsin and chymotrypsin, and bile release was simulated by addition of bovine bile extract. Release of pancreatin was not simulated because on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) it elutes close to the lipase under investigation. Sampling was conducted at 60, 120 and 180 min of the small intestinal phase.

Prior to conducting the assay, the enzyme eluted on SDS-PAGE as a single band just above 25 kDa. It was degraded if spiked into stomach medium which contained pepsin, but no other

[5] The E-value is the number of matches expected by chance when comparing a sequence with a database. It helps determine if the matches that are seen represent meaningful homology.

components of the stomach or small intestinal media interfered with the elution of the enzyme.

Under the conditions of the assay, the enzyme was completely degraded at the 120 min (2 h) sampling timepoint of the gastric phase, and therefore could not be detected at any sampling time-point of the small intestinal phase.

These results are relevant to the triacylglycerol lipase that is the subject of this application because expression by a different microorganism would not be expected to impact the digestibility of the enzyme.

3.3.4 Toxicology data

No genotoxicity assays or studies in laboratory animals have been conducted with the enzyme preparation that is the subject of this application.

3.3.5 Potential for allergenicity

A recent (2023) sequence homology search was conducted using the AllergenOnline database version 22 and FASTA36. The database was searched using a sliding window of 80-amino acids sequences derived from the full-length amino acid sequence, and a further search was conducted for an exact 8 amino acid match. No matches to known allergens were identified by either search strategy.

3.3.6 Assessments by other regulatory agencies

The specific triacylglycerol lipase that is the subject of this application has not yet been assessed by any other regulatory agency.

3.4 Dietary exposure assessment

The objective of the dietary exposure assessment was to review the budget method calculation presented by the applicant as a 'worst-case scenario' approach to estimating likely levels of dietary exposure, assuming that all of the TOS from the triacylglycerol lipase preparation remained in the food.

The budget method is a valid screening tool for estimating the theoretical maximum daily intake (TMDI) of a food additive (Douglass *et al* 1997). The calculation is based on physiological food and liquid requirements, the food additive concentration in foods and beverages, and the proportion of foods and beverages that may contain the food additive. Whilst the budget method was originally developed for use in assessing food additives, it is also appropriate to use for estimating the TMDI for processing aids (FAO/WHO 2020b). The method is used by international regulatory bodies and the FAO/WHO Joint Expert Committee on Food Additives (JECFA) (FAO/WHO 2021) for dietary exposure assessments for processing aids.

In their budget method calculation (the original calculation was revised during the assessment), the applicant made the following assumptions:

- the maximum physiological requirement for solid food (including milk) is 50 g/kg body weight/day
- 25% of solid food is processed
- all solid foods (bread and bakery products) contain the enzyme at the maximal recommended use level of 10.16 mg TOS/kg in the raw material (flour)

- all of the TOS from the enzyme preparation remains in the final products
- beverages were not included in this calculation since the proposed uses of the lipase is specific to solid food (bread and bakery products).

Based on these assumptions, the applicant calculated the TMDI of the TOS from the enzyme preparation to be 0.127 mg TOS/kg bw/day.

Assumptions made by the applicant do not differ from those that FSANZ would have made in applying the budget method that are conservative and reflective of a first tier in estimating dietary exposure. Hence, it was not required to recalculate the TMDI using different assumptions.

The applicant's estimates of the TMDI will be overestimates of the dietary exposure given the conservatisms in the budget method. This includes that it was assumed that all of the TOS from the enzyme preparation remains in the final foods. Whereas the applicant has stated that the enzyme is denatured by heat during the baking step and has no further technological function after baking.

4 Discussion and Conclusion

FSANZ concludes that the use of this triacylglycerol lipase as a processing aid for use in bread and bakery products is consistent with its typical function of catalysing the hydrolysis of triglyceride ester bonds and also acting on phospholipids and galactolipids, converting them to more efficient emulsifying structures. The use of triacylglycerol lipase improves crumb structure and bread volume in bread and bakery products. It is functioning as a processing aid for the purposes of the Code where it is does not perform a technological purpose in the food for sale. FSANZ also concludes that the evidence presented to support its proposed use provides adequate assurance that the use of the enzyme, in the quantity and form proposed to be used (which must be consistent with GMP), is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

K. phaffii has a long history of safe use as a production microorganism of enzyme processing aids. The production organism is neither pathogenic nor toxigenic. Analysis of the genetically modified production strain confirmed the presence and stability of the inserted DNA.

Sufficient information has been provided to assess the safety of the triacylglycerol lipase that is the subject of this application. While a history of safe use for this specific enzyme has not been established, the production organism itself has a long history of safe use and raises no issues regarding the presence of secondary metabolites of toxicological concern in the enzyme preparation. The enzyme itself is rapidly destroyed under conditions replicating those in the human stomach and duodenum, and no significant homology between the enzyme and any known toxins or allergens was identified.

Based on the safety assessment and considering the applicant calculated TMDI of the total organic solids (TOS) from the triacylglycerol lipase preparation (0.127 mg TOS/kg bw/day), no public health and safety concerns were identified in the assessment of the triacylglycerol lipase produced by this GM *K. phaffii* under the proposed conditions.

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