



SUPPORTING DOCUMENT 1

RISK ASSESSMENT REPORT

SUMMARY

The *Australia New Zealand Food Standards Code* (the Code) currently permits the use of numerous microbial enzymes as processing aids in the manufacture of food. Approval for lipase (EC 3.1.1.3) from a number of sources already exists in the Code.

Application A1036 seeks approval for the use of lipase derived from a genetically modified *Aspergillus niger*, as a processing aid. This strain of *Aspergillus niger* was modified using recombinant DNA techniques to contain multiple copies of the gene for an engineered form of lipase from *Fusarium culmorum*. The engineered lipase gene derived from *F. culmorum* contains the lipase gene sequences of several *Fusarium* species, as well as several changes unique to the current lipase.

The risk assessment has considered the technological suitability, the safety and identity of the donor and host microorganisms, and safety of the lipase enzyme preparation. Based on the available data, no food safety concerns have been identified with the enzyme, or with the donor or host organisms used to produce the enzyme, which would preclude permitting its use as a food processing aid. The absence of any specific hazards being identified is consistent with lipase undergoing normal proteolytic digestion in the gastrointestinal tract.

The stated purpose for the use of this lipase is to improve the gas holding capacity of dough in bread making. The Application provides adequate assurance that the lipase, in the form and amounts prescribed, is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

The available data are considered sufficient to provide an acceptable level of confidence in the conclusions of this risk assessment in regard to the safety and suitability of this lipase for its stated purpose.

Conclusions

- The use of *Aspergillus niger* as the host organism, is a well-characterised expression system for the production of enzymes, and has a long history of safe use.
- Enzymes from *Fusarium* species are generally considered to be safe, and several other *Fusarium* lipases have been approved for use by FSANZ.
- The evidence shows that this recombinant lipase is likely to be proteolytically degraded in the human gastrointestinal tract.
- There was no evidence of toxicity at any of the high doses tested in a 90-day repeat dose study. The No Observable Adverse Effect Level (NOAEL) was 2135 mg/kg bw/day, the highest dose tested. There was also no evidence of genotoxicity.

- Based on the reviewed toxicological data it was concluded that in the absence of any identifiable hazard a ADI (Acceptable Daily Intake) does not need to be specified.
- There is no evidence of any mycotoxins associated with the enzyme preparation.
- Based on the available evidence, lipase produced in *A. niger* is considered safe for use in foods for human consumption.
- The stated purpose for this lipase is to improve the gas holding capacity of dough for bread making. When used in the form and amounts prescribed, lipase is technologically justified and achieves its stated purpose.
- The lipase enzyme produced from the genetically modified *A. niger* described in this Application meets international specifications for identity and purity.

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1 Introduction

An application was received from DSM on 5 October 2009 seeking approval to permit a lipase produced from *A. niger* expressing a gene for a protein engineered variant of lipase produced from *Fusarium culmorum*, in Table to Clause 17 of Standard 1.3.3 Food Processing Aids of the Australia New Zealand Food Standards Code. Approval for lipase from a number of other sources already exists in the Code. The proposed use of the lipase is in bakery applications where its technological function is to enhance the gas holding capacity of dough, leading to increased stability upon proofing.

1.1 Objectives of the Assessment

In proposing to amend the Code to include a lipase derived from a genetically modified (GM) *A. niger* as a processing aid, a pre-market assessment is required. The objectives of this risk assessment are to determine:

- What are the potential public health and safety concerns that would arise from the use of this lipase as a processing aid?
- Is the proposed purpose clearly stated and does the enzyme achieve its technological function in the quantity and form to be added?

1.2 Risk Assessment Questions

The following risk assessment questions have been developed to address the objectives of the assessment.

- Is the enzyme safe for the proposed use?
 - Are the donor and source organisms safe for producing this lipase?
 - Are there any potential allergenicity concerns with any components associated with the production process?
 - Does the lipase share homology with known allergens?
- Does the enzyme achieve its stated technological purpose?
 - Is the quantity and form proposed for use, consistent with proposed use?

2 Characterisation of the Lipase

2.1 Identity of the enzyme

Systematic name:	Triacylglycerol acylhydrolase
IUBMB Enzyme Nomenclature:	EC 3.1.1.3
C.A.S number:	9001-62-1
Common name:	Lipase
Other names:	Triacylglycerol lipase, triglyceride lipase; tributyrase;

butyrylase; glycerol ester hydrolase; tributyrinase

Marketing Name: Panamore Golden

2.2 Chemical and physical properties

The lipase in this application is synthesised as a 347 amino acid pre-pro-lipase. Signal peptidase proteolytic digestion at both the N- and C-termini results in the mature protein of 274 amino acids.

2.2.1 Enzymatic properties

Lipase hydrolyses ester bonds of triacylglycerol to release free fatty acids from the glycerol backbone. It belongs to the subclass of carboxylic ester hydrolases,

The lipase described in this Application hydrolyses the following reaction:

Triacylglycerol + H₂O → diacylglycerol + a carboxylate

Apart from the above reaction, the lipase can also act on ester bonds of other lipid substrates, including (polar) diacyl lipids, phospholipids and glycolipids, such as galactolipids. Depending on the lipids present in the application, one of the above activities will be more prevalent than the other.

Lipase activity is determined spectrophotometrically using a chromogenic substrate; p-nitrophenyl-palmitate. Lipase hydrolyses this substrate into p-nitrophenyl and palmitic acid with the liberation of p-nitrophenyl, determined by measuring the increase of absorption at 405 nm over time, corresponding to lipase activity.

The activity, measured in DSM lipase units (DLU), is defined as the amount of enzyme that liberates 1 micromole p-nitrophenol per minute under the conditions described for the assay (Annex II.B.3-1 in the application).

The final granulated enzyme preparation has an activity of 2750 DLU/g.

The Applicant provided pH profile and temperature curve analysis for the lipase. Enzyme activity remained fairly constant (approximately 90% relative activity) over the pH range 6-9 at 37 °C, whilst the temperature optimum was found to be between 20-30 °C at pH 8.5. Activity rapidly declined at temperatures greater than 40 °C with no detectable activity at temperatures over 50 °C.

A theoretical molecular weight for the protein component of the constructed enzyme was calculated to be 28.7 kDa. The amino acid sequence of the recombinant lipase revealed four potential N-linked glycosylation motifs. SDS-PAGE analysis revealed four bands between 29 and 41 kDa. Deglycosylation with PNGaseF resulted in a single band of approximately 29 kDa, which is in good concordance with the calculated molecular weight. The isoelectric point of the glycosylated enzyme (ultrafiltration concentrate sample) was determined to be 4.5, with minor bands visible in a larger range (Annex II.B.3-2 in the application).

2.2.2 Physical properties

The commercial powder product is granulate in nature of which < 5% is smaller than 63 µm and > 90% is between 63 and 224 µm.

The Total Organic Solids (TOS) of the lipase preparation was calculated from three different batches of the ultrafiltrated (UF) concentrate. Considering 10% of enzyme activity is lost during spray-drying and the dilution factor associated with standardisation, the final enzyme preparation (2750 DLU/g) will have a TOS value between 42 and 47 mg/g enzyme preparation.

2.3 Production of the Lipase

The lipase is produced by a submerged fermentation process using appropriate substrates and nutrients followed by several filtration and purification steps. The fermentation process consists of two steps: inoculum fermentation and main fermentation. Biosynthesis and excretion of the lipase by the production organism occurs during the main fermentation phase. Once fermentation is stopped, the production organism is killed off using a validated procedure. The lipase is then separated from the fermentation broth through several simple filtration steps and concentrated by ultrafiltration. The UF concentrate is then spray dried in the presence of wheat flour and subsequently blended with granulated wheat flour to the desired lipase activity.

The fermentation process is carried out using Good Manufacturing Practice.

2.3.1 Description of the genetic modification

The production organism for the lipase is *A. niger* strain LFS54, which was derived by recombinant DNA methods from *A. niger* strain ISO-528. The genetic modification involved the exploitation of engineered “plug-sites” (defined, pre-inserted transgene integration sites) that had been previously introduced into *A. niger* strain GAM-53 (see Figure 1). A modified version of the *F. culmorum* triacylglycerol gene was inserted into the chromosome of ISO-528. The engineered form of the enzyme was altered in order to be more readily expressed in *A. niger*, as well as to increase its solubility for down-stream processing.

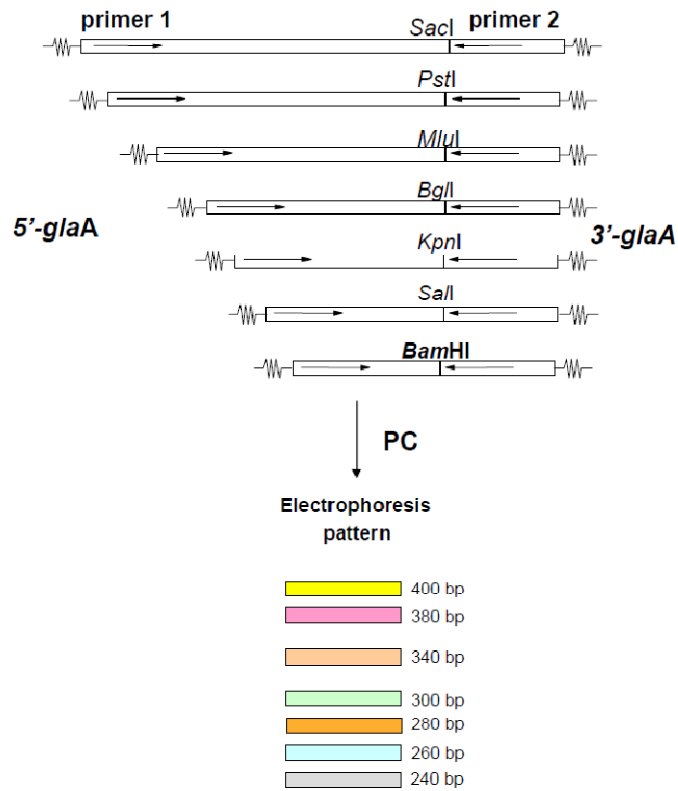


Figure 1: The “plug-in” sites for genetic modification of *A. niger* strain ISO-528 and their visualisation. Seven sites were introduced into *A. niger* strain GAM-53. Each site contains the *A. niger glaA* promoter and terminator as well as unique restriction sites. PCR amplification using two primers gives rise to seven unique bands. Insertion of a gene of interest disrupts this pattern, and thus location of the inserted gene can be determined. These PCR primer sites are designated “DNA Flags”.

The inserted gene is under the regulation of a native *A. niger* promoter and terminator. Initial steps of the genetic modification involve the linearisation and purification of the plasmids (resulting in removal of all *E. coli* sequences) and insertion of both lipase expression and selectable marker cassettes into the *A. niger* genome. Subsequent steps result in the complete removal of the selectable marker (see Figure 2). In order to increase expression of the lipase gene to commercially viable levels, the incidence of naturally-occurring duplication events was used to select for variants containing multiple copies of the lipase cassette (see Figure 3). The final production strain therefore contains only the integration cassette consisting of the engineered form of the lipase gene, but present as multiple copies.

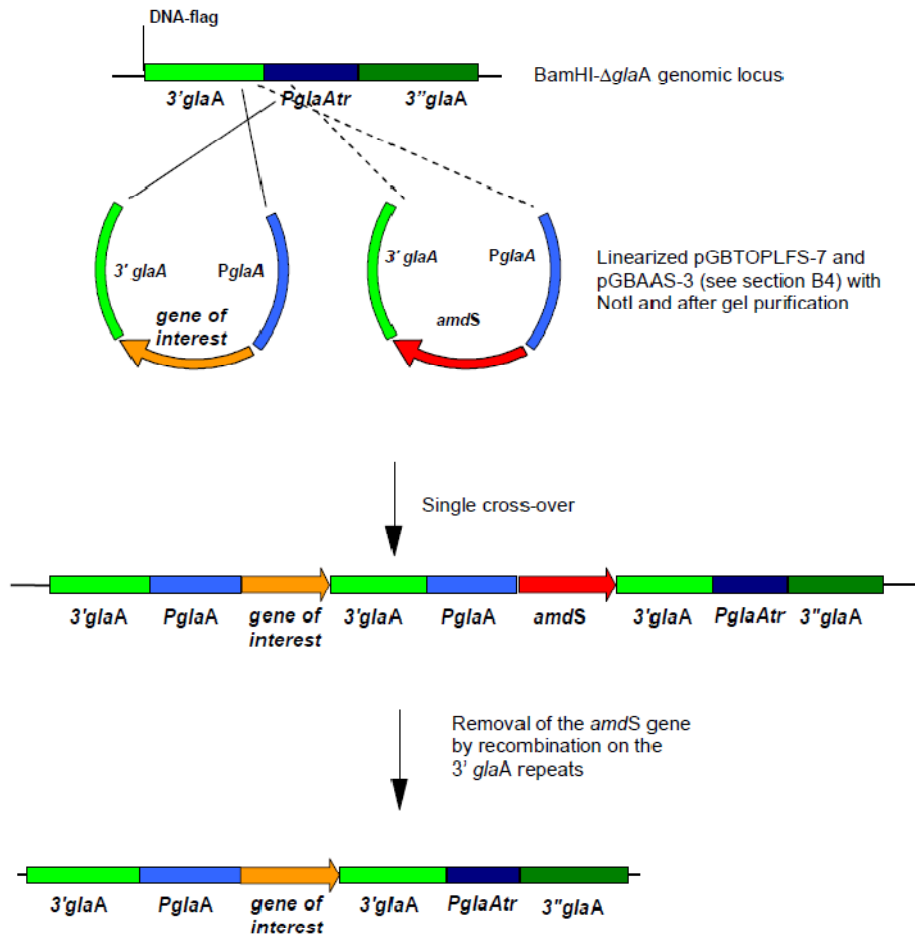


Figure 2: Schematic representation of marker-gene free insertion of an expression unit. Initially linearised expression cassettes containing *A. niger glaA* promoter and terminator sequences, with either the gene of interest (in this case triacylglycerol lipase) or the selection marker (*amdS*) are introduced by homologous recombination into a “plug site” (see Figure 1). Negative selection on fluoro-acetamide leads to selection for a transformant containing the desired lipase gene but without the selectable marker.

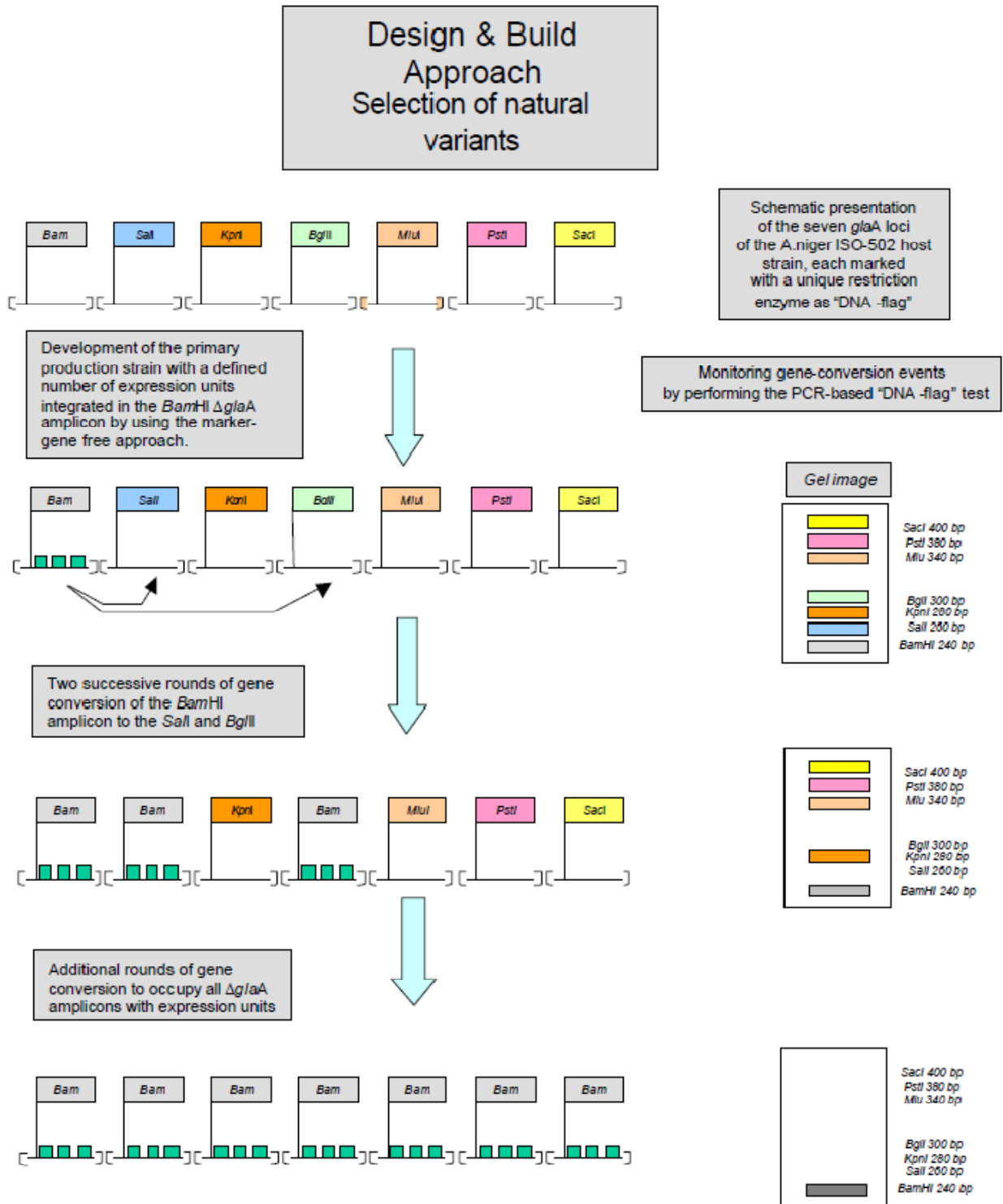


Figure 3: Strategy used for increasing transgene expression in *A. niger* to produce strain LFS-54. After initial selection and counter-selection (see Figure 2), the marker-free strain undergoes "gene conversion" (Selten *et al.*, 1998). This is detected by amplification of the "DNA-flag".

2.3.2 Identification of the donor and host organisms

a. Host strain

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 toxigenic potential of the production organism, that is, the possible synthesis by the production strain of toxins, and the potential for the carryover of these into the enzyme preparation (Pariza and Johnson, 2001).

A. niger is a fungus that is commonly found on fruits, seeds, nuts and vegetables. It is also frequently present as a contaminant of food. It is present as a black mould on susceptible hosts, and is considered to be a plant pathogen. Under optimal conditions, *A. niger* can cause systemic disease in some plants.

A. niger is not considered to be a human pathogen. Although inhalation of large quantities of spores can cause disease, *A. niger* has a history over several decades of safe use as a production organism for food enzymes. It is also a permitted source of a number of enzymes in the Code¹. A number of enzymes produced in *A. niger* have been evaluated for safety by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) and are considered to be non-toxic. No acceptable daily intake (ADI) for these enzymes has been specified. In addition, the US FDA has affirmed several enzyme preparations from *A. niger* as Generally Recognised as Safe (GRAS), and summarised the safety of *A. niger* as a host for enzyme expression (Olempska-Beer *et al.*, 2006).

b. Donor strain

The modified lipase is encoded by a novel gene sequence derived from a number of lipase genes from the fungal genus *Fusarium*. The primary homology is to the lipase gene of *F. culmorum*. However, the lipase from *F. culmorum* and the lipase in this application share approximately 82% amino acid identity. The expressed recombinant protein contains small interspersed peptide sequences of up to four amino acids which appear to be unique to this GM lipase. Thus it appears that the lipase gene from *F. culmorum* has been optimised for performance in bakery applications using specific mutations. If, instead, the sequence had contained a large block of sequence from another organism (for example, a domain), then it could be argued that there were two “donor organisms.” This not being the case, it seems appropriate to deem this lipase to have come from *F. culmorum*.

F. culmorum is a mould. *Fusarium* species are commonly found in soil and are pathogenic in plants. *F. culmorum* is able to produce a number of substances that can cause adverse health effects in humans including mycotoxins (Fung and Clark, 2004). 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*. Eight genes involved in the mycotoxin biosynthesis from *F. sporotrichioides* have been characterised, however none of these genes encodes a lipase enzyme. Although *F. culmorum* is known to produce mycotoxins (for example, deoxynivalenol, nivalenol and zearalenone) (Wagacha and Muthomi, 2007), none of these biosynthetic pathways involves a lipase.

¹ The following enzymes sourced from *A. oryzae* are permitted in the Code: aminopeptidase; α -amylase; carboxyl proteinase; β -glucanase; glucoamylase; α -glucosidase; xylanase; lactase β – galactosidase; triacylglycerol lipase; metalloproteinase; mucorpepsin; pectin methyltransferase; 6-phytase; polygalacturonase; serine proteinase; and phospholipase A1.

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. antophyllum*, *F. proliferatum* and *F. chlamydosporum*. However, systemic infection has not been reported with *F. heterosporum*, one of the sources of sequence for the lipase gene used for transformation of the production organism.

2.4 Analysis and Specifications

2.4.1 Methods of analysis

A method for determining the activity of this lipase in fermentation samples, downstream processing samples and end product concentrates has been provided by the Applicant (Annex II.B.3-1 in the application).

No specific methodology has been supplied for determination of lipase activity in the final food product, namely bakery products; however the methodology provided for assessing enzyme concentrate was used to assess the presence of residual enzyme activity in baked bread for the inactivation trials carried out by the Applicant. It should be noted that dosage levels used in the analysis were far greater (1415-2122x higher) than would normally be used for commercial use.

2.4.2 Specifications

The Applicant states specifications written for this lipase comply with the international specifications for identity and purity relevant for enzymes prepared by the FAO/WHO Expert Committee on Food Additives (JECFA, 2006). These specifications are primary reference sources listed in Clause 2 of Standard 1.3.4 - Identity and Purity, of the Code.

Specifications for the commercial product, as provided by the Applicant, are described in the below table. Three Certificates of Analysis for the commercial enzyme preparation have been provided by the Applicant which demonstrate conformance to the stated specifications (Annex II.B.5-1 in the application).

Lipase produced from a genetically modified *A. niger* expressing a gene based on the pre-pro-lipase encoding gene sequences of various *Fusarium* species, meets international specifications for identity and purity.

Table 1: Specifications for the commercial lipase enzyme preparation (as provided by the Applicant)

	Specification
Lipase activity	2750 ± 10% DLU/g
Appearance	Off-white to creamy granulates
Particle size	63 µm < 90% < 224 µm
Dry matter	> 90%

	Specification
Cadmium	≤ 0.5 mg/kg
Mercury	≤ 0.5 mg/kg
Arsenic (as As)	≤ 3 mg/kg
Lead	≤5 mg/kg
Standard Plate Count	≤5 x 10 ⁴ cfu/ml
Coliforms	≤30 cfu/ml
Salmonella	Absent in 25 g
E. coli	Absent in 25 g
Anaerobe sulphite reducing	< 30 cfu/g
Staphylococcus aureus	Absent in 1 g
Antibiotic activity	Negative by test
Mycotoxins	Absent by test

3 Technological function of the enzyme

The lipase's technological effect in bakery applications is to enhance the gas holding capacity of the dough leading to increased stability of the dough upon proofing. This translates to an increased loaf volume; improved loaf shape and oven spring post-baking. Further effects are improved crumb structure and softness. Further, the Applicant states that using this lipase will reduce or eliminate bread manufacturers' reliance on flour/bread improvers to deal with seasonal variations of flour.

The mechanisms underlying these technological effects are mainly based on the generation of polar lipids from the lipids naturally present in the dough. The natural content of lipids in wheat flour is approximately 2.5% (w/w), comprising both polar and apolar lipids. The gas holding capacity of dough is highly influenced by the lipid composition of the flour. The higher the content of highly polar monoacyl lipids, the better the gas-holding capacity and thus the baking performance will be.

The lipase in question can be used to convert less polar, less emulsifying diacyl phosphor- and galactolipids present in the flour to their highly polar, stronger emulsifying monoacyl counterparts. In addition, the lipase can also convert apolar triglycerides, present in the flour or added fat, to relatively more polar di- and monoglycerides which results in increased crumb softness and regularity.

The average dosage of the enzyme depends on the application, the type and quality of the raw materials used and the process conditions. The suggested dosage range for this lipase preparation is between 14 DLU/kg and 164 DLU/kg flour, with a more frequently recommended range of 27-77 DLU/kg flour.

Various dosages of the lipase were assessed by the Applicant for the production of 'Batard' and 'Sandwich' type bread. Dosages used range from 11.5 to 27.5 DLU/kg flour in Batard

bread to 27.5 to 82.5 DLU/kg flour in Sandwich bread. The effect of the lipase on loaf volume was measured by an automated bread volume analyser whilst effects on dough stability, oven spring and crumb structure were assessed visually by a suitably experienced baker. Results of the baking performance analysis clearly indicated that addition of this lipase improved dough stability, oven spring and crumb regularity and enhanced loaf volume when compared to the controls (Annex II.G.1-1 in the application). Use level and efficacy of the enzyme depends on the type of bread being produced and is therefore influenced by the type and quality of flour used. Both baking trials conducted by the Applicant demonstrate the lipase enzyme's effectiveness across a range of dosage levels and bread types.

In all baking applications, the action of lipase takes place during mixing and proofing and thus prior to baking. Inactivation trials conducted by the Applicant confirms that the enzyme is inactivated during the baking process (Annex II.G.2-1 in the application). The inactivation trial results support the temperature profile data supplied by the Applicant. No further reaction products are expected to be formed during storage of the bread and the inactivated enzyme will remain inert in the food as any other protein.

3.1 Conclusion

The stated purpose for this lipase, namely to improve the gas holding capacity of the dough is clearly articulated in the Application and the baking trial and inactivation evidence presented in support of the Application, provides adequate assurance that the lipase, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

4 Safety Assessment

The following studies were evaluated as part of the hazard assessment:

- A digestibility study
- An assay for toxic metabolites
- 14-day dose range-finding oral toxicity study in rats
- 90 day oral (diet) toxicity study in rats
- Bacterial reverse mutation assay (Ames test)
- *In vitro* mammalian chromosomal aberration test with human lymphocytes
- Biochemical characterization of the lipase

4.1 Digestibility studies

M. Misset (2007). Analysis of the pepsin resistance/sensitivity of the lipase LFS7 (A18356). DSM R&D/Analysis. Delft, The Netherlands.
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This study was undertaken to determine the *in vitro* susceptibility of lipase to pepsin.

Recombinant lipase, as well as an appropriate pepsin-resistant control protein and an unrelated pepsin-sensitive control were subjected to digestion over 60 minutes in a 0.32% pepsin solution. As anticipated, the pepsin-resistant β -lactoglobulin control survived the pepsin treatment. In contrast, the pepsin sensitive control (lipoxidase) was hydrolysed within 30 seconds to small peptides which could not be detected by protein staining under reducing or non-reducing conditions on SDS-PAGE. For untreated recombinant lipase, there were three stained bands with one co-migrating with deglycosylated lipase. One possible explanation is that, at most, three of the four potential N-linked glycosylation motifs were occupied. An alternative explanation is that only one or two sites are glycosylated but the

carbohydrate chain length differs at the glycosylation site/s. Unfortunately, as pepsin co-migrated with one of the three stained lipase bands on SDS-PAGE the extent of digestion could not be unequivocally determined. However, as the three other glycosylated recombinant lipase proteins were no longer visible and the recombinant lipase amino acid sequence suggested susceptibility to pepsin hydrolysis it was presumed that digestion to short peptides was complete.

4.2 Toxicity studies

The test material used in all the toxicity studies was spray dried with maltodextrin in two portions (KPF0726A/FUR/A and KPF0726A/FUR/B). These lipase preparations had an enzyme activity of 9550 DLU/g, and total organic solids (TOS) contents of 47.2 and 46.7% respectively, which is typical of food grade preparations.

4.2.1 Short-term toxicity

B.A.R. Lina (2007). 14-day dose range-finding/feasibility study with an enzyme preparation of *Aspergillus niger* containing lipase activity (LFS528-24A) in rats. TNO Food and Nutrition, Zeist, The Netherlands.

This preliminary limited study was only intended to yield information about appropriate doses to use in a 90-day oral toxicity study so consequently it was considered unnecessary to be GLP compliant.

The study consisted of four groups of four male and four female six week-old Wistar WU outbred rats. One group consumed the control diet (ie without added lipase preparation) and three groups received various doses of the recombinant lipase preparation in food. Test groups two, three and four received 0.4%, 1.2% and 3.6% recombinant lipase preparation in the normal diet, respectively.

Clinical observations were made twice daily on working days and once daily on weekends. Bodyweights were recorded on Day 0 and twice weekly thereafter. Food consumption was measured every 3-4 days, depending on the frequency of refreshing the feeders. Animals were killed at the end of the observation period and subjected to gross necropsy, the organs were examined and weighed and the stomach was sectioned at 5 µm and examined histologically.

No deaths were recorded and there were no clinical signs. All the animals showed normal food intake and bodyweight gain and no treatment-related abnormalities were noted at necropsy. There were no significant differences in organ weights between the test groups and the controls.

The conclusion of the study was that a diet containing up to 3.6% lipase was well-tolerated by the rats. It was therefore decided that dietary levels of 0.4%, 1.2% and 3.6% lipase would be tested in a subsequent 13-week study.

B.A.R. Lina (2008). Subchronic (13-week) dose range-finding/feasibility study with an enzyme preparation of *Aspergillus niger* containing lipase activity (LFS528-24A) in rats V7712. TNO Quality of Life, Zeist, The Netherlands.

A GLP compliant study was conducted according to OECD Test Guideline 408 (Repeated Dose 90-day Oral Toxicity Study in Rodents (1998)) and EC guideline 2001/59/EC (B.26: Sub chronic oral toxicity test: Repeated dose 90-day oral toxicity study in rodents). The study

consisted of four groups of ten male and ten female six week-old Wistar WU outbred rats. One group consumed the control diet and three groups received various doses of the lipase preparation in food. Test groups two, three and four received 0.4%, 1.2% and 3.6% lipase preparation in the normal diet, respectively.

Clinical observations were made twice daily on working days and once daily on weekends and public holidays. Bodyweights were recorded on Day 0 and once per week thereafter. Food consumption was measured every 3-4 days, depending on the frequency of refreshing the feeders. Water consumption was measured daily (at least 5 days/week) in weeks 1, 6 and 12. Neurobehavioural testing (functional observational battery tests, including grip strength testing and sensory reactivity to stimuli testing) was conducted prior to first exposure and then once weekly. Ophthalmoscopic observations were made prior to treatment and then on day 90 in the control and high-dose group only. Haematology was conducted at the end of the treatment period, as was clinical chemistry. Urinalysis was conducted on day 84-85. Organs were weighed and examined microscopically along with other tissues.

No rats died during the study. No treatment-related clinical signs were observed. There were no behavioural observations that would indicate neurotoxic potential during the study period. There were no treatment-related ophthalmic changes. There were no statistically significant differences in body weight gain or overall mean food intake. There were no significant differences in haematology or clinical chemistry or urinalysis between the control and test groups. There were no treatment-related differences in gross necropsy or at a histopathological level. Of the organs, the spleen showed a significant increase in size (approximately 8%) in males only in the high-dose test group. As there were no significant changes in measured haematological parameters or corroborating histopathological lesions in the spleen the biological significance of this observation is equivocal.

Overall, a diet containing up to 3.6% lipase preparation was well tolerated. The 'No Observed Adverse Effect Level' (NOAEL) in this study was considered to be 3.6% of the diet. Based on food consumption, this corresponds to 2135 mg/kg bw (1008 mg TOS or 20389 DLU) for males and 2250 mg/kg bw/day (1062 mg TOS or 21487 DLU) for females.

4.2.2 Genotoxicity

The results of two *in vitro* genotoxicity studies with recombinant lipase are summarised below. Both studies were GLP compliant and conducted in accordance with OECD Test Guidelines 471 (Bacterial Reverse Mutation Test) and 473 (*In vitro* Mammalian Chromosome Aberration Test). Neither test revealed any genotoxic potential associated with recombinant lipase.

4.2.2.1 Reverse mutation test (Ames test)

M.J.M. van den Wijngaard (2008). Bacterial reverse mutation test with enzyme preparation of *Aspergillus niger* containing lipase activity (LFS528-24A). TNO Quality of Life, Zeist, The Netherlands.

A bacterial reverse mutation test (Ames test) was conducted with the same preparation of enzyme as described above to determine if the recombinant lipase preparation as prepared from *A. niger* has mutagenic potential. This test was done using four strains of *Salmonella typhimurium* (TA 1535, TA 1537, TA 98 and TA 100) as well as the *Escherichia coli* mutant WP2 *uvrA*. The tests were carried out in the presence or absence of rat hepatic microsomal fraction S9. Three tests were carried out as described:

In the first test (plate-incorporation method), all strains were tested in the presence and absence of S9-mix. Six concentrations were tested (0 µg, 62 µg, 185 µg, 556 µg, 1667 µg, 5000 µg TOS/plate). The first test was performed twice for three strains without S9-mix as the positive or negative controls were not within historical control values. In this test, and increase of up to two-fold was observed in the mean number of revertants in strains TA 1535, TA 1537 and TA98 in the presence and absence of S9-mix. This was associated with a slightly more dense background lawn. This was attributed to the proteinaceous nature of the test substance. Therefore a second test was performed using a different method.

The second test was performed using the treat and plate method. In this test, concentrations of the substance above 131 µg TOS/ µl were toxic to strains TA 1535 and TA 98 in the absence of S9-mix and to strain TA 1537 both with and without S9-mix. Fewer than three non-toxic dose levels could be evaluated, therefore a third test using the treat and plate method was carried out using TA 1535, TA 1537 and TA 98.

In the third test, the lipase preparation was toxic in concentrations above 167 µg TOS/ µl in the absence of S9-mix to strains TA 1535 and TA 98. The lipase was toxic in concentrations above 56 µg TOS/ µl to strain TA 1537 in the absence of S9-mix.

The second and third tests did not show any dose-related or more than two-fold increase in the mean number of revertant colonies. It was concluded that the tox-batch was not mutagenic under the conditions used in the study.

4.2.2.2 Chromosomal aberration test

B. Usta and N. de Vogel (2008). Chromosomal aberration test with enzyme preparation of *Aspergillus niger* containing lipase activity (LFS528-24A) in cultured human lymphocytes. TNO Food & Nutrition, Zeist, The Netherlands.

The enzyme preparation containing *A. niger*-derived recombinant lipase was tested for its potential to induce chromosomal aberrations in cultured human lymphocytes. This was done in the presence or absence of S9-mix.

Two tests were conducted, each with lymphocytes cultured from a different donor. In the first test, the lymphocytes were incubated with 0, 39, 78, 156, 313, 625, 1250, 2500 or 5000 µg/ml of the enzyme preparation for four hours in the presence or absence of S9-mix, before being incubated for an additional 20 hours in fresh medium. After fixation, 200 cells per concentration were analysed microscopically for aberrations/anomalies. The higher concentrations (625-5000 µg/ml) in the presence of S9-mix were cytotoxic and thus no cells could be observed. This was true also of the 2500 and 5000 µg/ml concentrations in the absence of S9-mix. At the observable concentrations, no statistically significant increase in the number of aberrant cells was observed at any concentration.

In the second test, cells were treated differently in the presence or absence of S9-mix. In the presence of S9-mix, the final concentrations of the enzyme preparation were 50, 100, 150, 200, 300, 400, 500 and 600 µg/ml. The cells were exposed to the test compound for 4 hours, before being harvested 24 hours after onset of treatment. Concentrations of 300 µg/ml and above were cytotoxic and thus disregarded. Of those concentrations tested, none was associated with a statistically significant increase in the number of aberrant cells.

In the absence of S9-mix, the concentrations tested were: 6.25, 12.5, 25, 50, 150, 300, 500, 750, 1000, 1500, 1750, 2000, 2250 and 2500 µg/ml. In this case, the cells were incubated continuously in the presence of the enzyme preparation for 24 hours. Only cells incubated in

the presence of 300 µg/ml and under were able to be analysed. At these concentrations, no statistically significant increases in the number of aberrant cells were observed.

The conclusion drawn from the study was that the enzyme preparation from *A. niger* was cytotoxic but not clastogenic to cultured human lymphocytes.

4.3 Production strain analyses

4.3.1 Analysis for toxin production

R.A. Samson & I.J. Houbraeken (2007). Analyses of one fermentation broth and one UF concentrate. Fungal Biodiversity Centre, Utrecht, The Netherlands.

R.A. Samson & I.J. Houbraeken (2008). Analyses of one *Aspergillus* strain on the presence of toxic metabolites. Fungal Biodiversity Centre, Utrecht, The Netherlands.

Some strains of *Aspergillus* are capable of producing toxins that are harmful to human health (Bennett, 1987; Coenen and Aughton, 1998; Cole and Cox, 1981). In their first examination, Samson and Houbraeken extracted samples from a fermentation broth and an ultrafiltration (UF) concentrate of the culture and sent them for mycotoxin testing. The extracts were analysed by high performance liquid chromatography (HPLC) with diode array detection (DAD). No relevant levels of mycotoxins were found in the sample fermentation broth or the UF concentrate. The authors noted that gluconic acid appeared to be present in the sample, which could have disguised the presence of small quantities of other metabolites. However known *Aspergillus* metabolites, such as ochratoxins A, B and alpha, were not present.

Despite the absence of known secondary metabolites, the parental strain from which LFS54 was derived was tested for its potential to synthesize mycotoxins. To this end, strain DS55898, grown under conditions optimal for the production of secondary metabolites, was tested. These conditions would not be used under normal production, as there is an inverse relationship between the production of commercial enzymes (i.e. during “trophophase”) and the production of toxic, secondary metabolites (i.e. during “idiophase”) (Turner, 1971). The analysis revealed the characteristic pattern of secondary metabolites for *A. niger* strains, but no relevant levels of mycotoxins.

The absence of any detectable mycotoxins, even under conditions optimal for their expression, provides re-assurance that preparations containing recombinant lipase are non-toxic.

4.4 Conclusion

Following the safety assessment of lipase from *A. niger*, it is concluded that:

- There was no evidence of any toxicity in a 13-week oral toxicity study in rats. Based on food consumption, the NOAEL was 2135 mg/kg bw (1008 mg TOS or 20389 DLU) in males and 2250 mg/kg bw/day (1062 mg TOS or 21487 DLU) in females.
- There was no evidence of any genotoxicity;
- There was no evidence of any mycotoxin production associated with the enzyme preparation.

4.5 Potential allergenicity

4.5.1 Enzyme

The Applicant presented the results of a bioinformatic assessment of the lipase protein. In the first analysis, the lipase sequence was compared with the Allermatch database to identify sequences of 35% or greater homology with known allergens. No significant matches were found between this lipase and known allergens.

In a second analysis, the lipase sequence was compared with the Allermatch database to identify sequences of 6 contiguous amino acids with 100% identity to known allergens. The conventional practice is to compare 8 contiguous amino acids to reduce the number of false positives that occur with a comparison of 6 amino acids. In their search, the applicant identified only one 6 amino acid match, which matched to a potato protein. However, this sequence forms part of the signal sequence for secretion of lipase and is thus cleaved before secretion into the culture medium. It therefore forms no part of the enzyme preparation used in food applications and is not considered to be relevant.

Data indicates that the recombinant lipase present in LFS54 shares 79.4% amino acid sequence homology with the equivalent lipase in *Fusarium heterosporum* and 79.0% with that in *Fusarium venenatum*. The lipase from the former species has been approved in Australia/New Zealand and notified as GRAS (GRN000075) for use in baking applications. The latter species has been available as an edible protein source for human consumption since 1984 without ill effects.

4.5.2 Ingredients used in manufacture

To make the enzyme suitable for use in bakery products, the ultrafiltrate enzyme concentration is spray-dried in the presence of wheat flour and then standardised with granulated wheat flour to the desired enzyme activity. Use of wheat flour is commensurate with the intended use of the final enzyme preparation.

If approved, the final food product produced using this enzyme would be required to be labelled in accordance with the provisions set out in Standard 1.2.3 – Mandatory Warning and Advisory Statements and Declarations, for the declaration of cereals containing gluten.

4.6 JECFA consideration

JECFA has considered a number of *A. niger* enzymes. These enzymes have all received an ADI of “not specified” (JECFA, 1990). However, JECFA has not considered recombinant enzymes.

5 Dietary Exposure

Processing aids perform their technological function during the manufacture of food and are therefore either not present in the final food or present only at very low levels. The applicant has provided estimated daily intake (EDI) data for the lipase based on final food residual enzyme level data from their inactivation trials and 90th percentile food intake data from The Netherlands and USA (Section G4 in the application). The EDI was determined to be between 0.041-0.675 DLU/kg bw based on The Netherlands data and 0.039-0.65 DLU/kg bw using the US data. In the Australian and New Zealand situation, the EDI is likely to be closer to the US data, given our similar diet and food intake levels.

This lipase is expected to be inactivated during baking and have no further technical effect after baking. Any residual enzyme would be present as denatured protein and would

undergo normal proteolytic digestion in the gastrointestinal tract.

FSANZ accepts the submitted dietary exposure evidence and this together with no ADI supports the determination that further dietary exposure assessment is unnecessary.

6 Response to Risk Assessment Questions

Is the enzyme safe for the proposed use?

The Safety Assessment reviewed evidence examining potential toxicity (90-day rat study) and genotoxicity associated with the enzyme preparation. There were no hazards identified, which would preclude permitting use of the enzyme as a food processing aid. The absence of any specific hazards being identified is consistent with the recombinant lipase undergoing normal proteolytic digestion in the gastrointestinal tract.

Are the donor and source organisms safe for producing this lipase?

The microbiological evidence assessed to establish the safety of the new genetically modified strain of *A. niger* included a review of the donor and host microorganisms and cytotoxicity screening tests. The host strain of *A. niger* has a long history of safe industrial use and is currently listed in the Code for the production of enzymes used as processing aids. Cytotoxicity tests using the ultrafiltrated concentrate were negative.

Are there any potential allergenicity concerns with any components associated with the production process?

Wheat flour is used to formulate the commercial enzyme preparation, hence the product triggers labelling provisions set forth in Standard 1.2.3 – Mandatory Warning and Advisory Statements and Declarations, for the declaration of cereals containing gluten.

Is there homology of the constructed lipase enzyme to known allergens?

The lipase sequence was compared with the Allermatch database. No sequence homology above 35% was identified between the lipase protein and any known allergens. In addition, no relevant matches of 6 contiguous amino acids were identified.

Does the enzyme achieve its stated technological purpose? Is the quantity and form proposed for addition, consistent with proposed use?

The Application clearly articulates the stated purpose for this lipase, namely to improve the gas holding capacity of the dough. The evidence submitted in support of the Application provides adequate assurance that the lipase, in the form and amounts added is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

7 Conclusion

The risk assessment has considered the technological suitability, the safety and identity of the donor and host microorganisms and safety of the lipase enzyme preparation.

The evidence presented was sufficient to determine that no toxicological or hazard-related concerns with the enzyme or the donor or host microorganisms exist. The absence of any specific hazards being identified is consistent with lipase undergoing normal proteolytic

digestion in the gastrointestinal tract. Thus lipase is safe for use as a food processing aid. The ADI for lipase is 'not specified'.

It was further concluded that the proposed use of the enzyme, namely to improve the gas holding capacity of the dough in bread making applications, was technologically justified in the form and prescribed amounts, and demonstrated to be effective.

8 References

Bennett, J.W. (1987) Mycotoxins, mycotoxicoses, mycotoxicology and Mycopathologia. *Mycopathologia* 100(1):3-5.

Coenen, T.M. and Aughton, P. (1998) Safety evaluation of amino peptidase enzyme preparation derived from *Aspergillus niger*. *Food Chem. Toxicol.* 36(9-10):781-789.

Cole, R.J. and Cox, R.H. (1981) *Handbook of Toxic Fungal Metabolites*. Academic Press, New York.

Fung, F. and Clark, R.F. (2004) Health effects of mycotoxins: a toxicological overview. *J.Toxicol.Clin.Toxicol.* 42(2):217-234.

JECFA (1990) *Joint FAO/WHO Expert Committee on Food Additives, 35th meeting, Rome, 29 May - 7 June 1989, "Enzyme preparations derived from Aspergillus niger."*. WHO Technical Report Series. Report No. 789.

JECFA (2006) *Compendium of Food Additive Specifications - General specifications and considerations for enzyme preparations used in food processing*. Joint FAO/WHO Expert Committee on Food Additives. FAO Food and Nutrition Paper Monograph 3., 67th session, 63-67. <ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf>. Accessed on 16 September 2008.

Olempska-Bier, Z.S., Merker, R.I., Ditto, M.D. and DiNovi, M.J. (2006) Food-processing enzymes from recombinant microorganisms--a review. *Regul.Toxicol.Pharmacol.* 45(2):144-158.

Pariza, M.W. and Johnson, E.A. (2001) Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. *Regul.Toxicol.Pharmacol.* 33(2):173-186.

Selten, G.C.M., Swinkels, B.W. and Bovenberg, R.A.L. (1998) Gene conversion as a tool for the construction of recombinant industrial filamentous fungi. WO 98/46772.

Sweeney, M.J. and Dobson, A.D.W. (1999) Molecular biology of mycotoxin biosynthesis. *FEMS Micrbiol Letts* 175(2):149-163.

Turner, W.B. (1971) *Fungal Metabolites*. Academic Press, New York.

Wagacha, J.M. and Muthomi, J.W. (2007) *Fusarium culmorum*: Infection process, mechanisms of mycotoxin production and their role in pathogenesis in wheat. *Crop Protection* 26(7):877-885.