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A Petition to Amend the Australia New Zealand Food Standards Code with an

Triacylglycerol Lipase Enzyme Preparation produced by *Trichoderma reesei*

EXECUTIVE SUMMARY

The present application seeks to schedule 18 - Processing Aids of the Australia New Zealand Food Standards Code (the Code) to approve an enzyme preparation from ***Trichoderma reesei*** *(T. reesei)* host strain genetically modified to produce a ***T. reesei*** **production strain (AR-822) containing a Triacylglycerol Lipase encoding gene** from *Thermomyces lanuginosus*. The enzyme is to be used in the manufacture of:

- bakery products such as, but not limited to bread, steamed bread, bread buns, tortillas, cakes, pancakes, and waffles
- Other cereal-based products

Proposed change to Standard 1.3.3 - Processing Aids

The table **Schedule 18—9(3) Permitted processing aids — various purposes (section 1.3.3—11)**, is proposed to be amended to include a genetically modified strain of *Trichoderma reesei* as permitted source for **Triacylglycerol Lipase** (EC 3.1.1.3).

This application is submitted under a general assessment procedure.

The food enzyme is a biological isolate of variable composition, containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process.

The main activity of the food enzyme is lipase.

Use of the Enzyme and Benefits

The main activity of the *Trichoderma reesei* AR-822 enzyme preparation is lipase (IUBMB 3.1.1.3). The **function** of lipase to catalyze the hydrolysis of ester bonds of triacylglycerols (at the position 1 and 3 of the glycerol molecule), resulting in the formation of mono- and diacylglycerols, free fatty acids and, in some cases, also glycerol.

The substrates for the lipase are non-polar lipids such as triglycerides or triacylglycerol. Triglycerides are formed by combining glycerol with three fatty acids molecules. The glycerol molecule has three hydroxyl (OH-) groups. Each fatty acid has a carboxyl group (COOH-). In triglycerides, the hydroxyl groups of the glycerol join the carboxyl groups of the fatty acid to form esters bonds.

Triglycerides are found in plants and animals: they are the main constituents of vegetable oils and animal fats. Triglycerides and triacylglycerols are also found for instance in wheat flour: wheat flour contains approximately 2.0–2.5% lipids; wheat lipids can be divided into glycolipids, phospholipids and non-polar lipids (triacylglycerides, mono-glycerides) as shown in Figure 1 below.

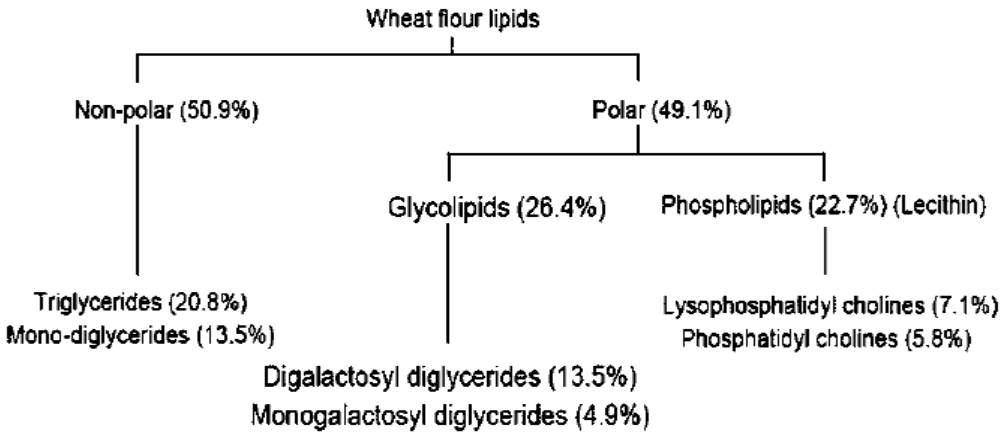

Figure 1: Classification of wheat flour lipids

The reaction products of the hydrolysis of non-polar lipids with the help of lipase are mainly mono- and diacylglycerols and free fatty acids. As the non-polar lipids containing organisms themselves produce lipases, these reaction products are naturally present in these organisms. Consequently, also the reaction products occur naturally in foods.

Lipase activity is widely present in nature and in food ingredients. The substrates and the reaction products are themselves present in food ingredients. No reaction products which could not be considered normal constituents of the diet are formed during the production or storage of the enzyme treated food. Consequently, no adverse effect on nutrients is expected.

The method to analyze the activity of the enzyme is company specific and is capable of quantifying lipase activity as defined by its IUBMB classification. The enzyme activity is usually reported in ALU/g.

Enzymatic function	catalyze the hydrolysis of ester bonds of triacylglycerols (at the position 1 and 3 of the glycerol molecule)
Substrates	non-polar lipids such as triglycerides or triacylglycerol
Reaction Products	mono- and diacylglycerols and free fatty acids

Like most of the enzymes, lipase performs its technological function during food processing. The lipase from *Trichoderma reesei* AR-822, subject of this dossier, acts as processing aid in the manufacture of bakery products (e.g., bread, biscuits, tortillas, cakes, steamed bread and croissants), and other cereal-based products (e.g., pasta, noodles and snacks). Lipases have been used in baking for the last 30 years and their use in the bakery industry is continuously increasing. This application has been specifically approved for a number of years in Canada, Denmark and France (including the "Pain de tradition Française"), USA, Mexico, Canada, Australia/New Zealand which together with the extensive use for decades demonstrates the technological need of lipases in these food processes.

Baking processes

In baking, lipase performs its technological function during dough or batter handling in order to contribute to an improved and consistent baking process. During mixing, wheat flour free lipids become bound or trapped within the gluten fraction. Limited hydrolysis of the triglycerides with the help of lipase results in an improved natural ratio of polar lipids. Increased proportion of polar lipids has a positive effect on gas retention, as they can align at the interface of the gas cells formed in the dough and therefore increase the stability of the gas cells, whereas endogenous wheat non-polar lipids destabilise gas cells in dough and therefore limit bread volume. The use of lipase helps removing this negative effect.

In addition, the degradation of the substrate triglycerides with the help of lipase leads to the creation of monoacyl-glycerol, that interacts with gelatinizing starch, in particular with amylose to form irreversible monoacyl-glycerol-amylose complexes.

The use of lipase can therefore influence the interactions between the different constituents of the dough, i.e., gluten proteins and lipids, starch and lipids as well as gluten and starch. The benefits of the conversion of triglycerides (non-polar lipids) with the help of lipase in baking can therefore be summarized as follows:

- Facilitate the handling of the dough (Colakoglu and Özkaya 2012)
- Improve dough stability and strength which results in processing tolerance (Rodríguez-García et al. 2014; Colakoglu and Özkaya 2012)
- Improve the dough's structure and behaviour during the baking steps (Rodríguez-García et al. 2014)
- Regulate batter viscosity, beneficial in the production process for e.g., waffles, pancakes and biscuits (Colakoglu and Özkaya 2012; Sîrbu and Pâslaru 2005; Ma et al. 2022)

The process flow of lipase used in the baking process is presented below:

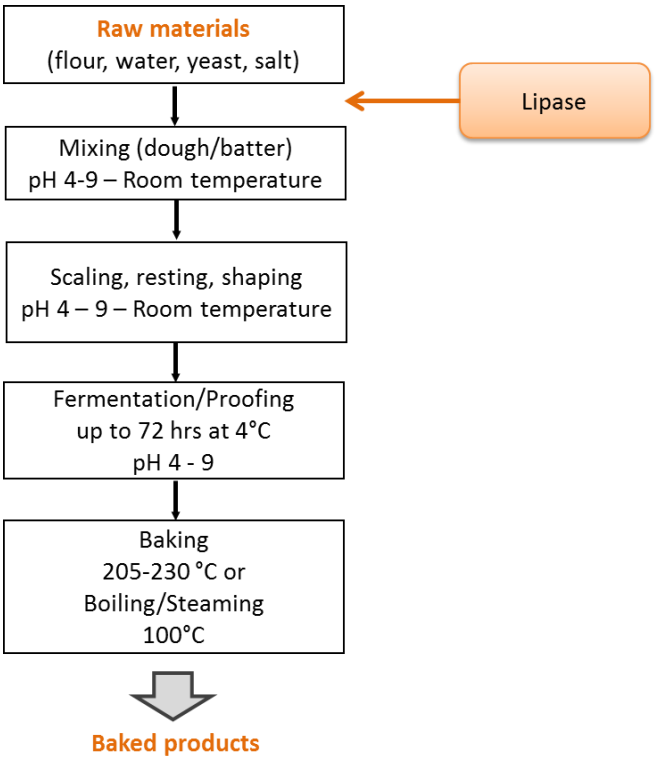

Figure 2: Lipase used in baking products

Other cereal-based processes:

Lipids provide functional properties during pasta, noodle and snack making - due to their ability to interact with gluten and the water phase. Limited hydrolysis of lipids with the help of lipase improves the functional properties of the flour endogenous lipids, as explained below.

Dried pasta has, among cereal derived foodstuffs, a very distinct microscopic structure. It has a continuous protein mixture phase (the gluten or the protein network) wherein the starch granules are dispersed. While cooking in hot water, the starch granules gelatinize, i.e., absorb water, swell and turn into starch paste. The gluten (the protein network) is denatured through cooking and if it is not sufficiently resistant, the starch granules, when swelling, can tear the meshes of its continuous phase, thereby giving rise, at the periphery of the pasta, to a viscous layer of starch paste.

The state of the protein network after cooking can also affect the elasticity of the pasta. The main problem which has to be solved to obtain elastic and non-sticky pasta thus consists in increasing the resistance of the protein network to cooking[1].

Pasta treated with lipase show higher amylose-lipid melting enthalpies (increase of around 75% more melting enthalpy in the cooked pasta treated by lipase), indicating that hydrolysis products of lipase do form complexes with amylose during cooking. These complexes inhibit the swelling of starch and the leakage of amylose during cooking, resulting in a firmer texture and smoother surface. Further, the complex-building capability of the lipase hydrolysis products with amylose reduces leaching of amylose, resulting in less stickiness of products[2].

Because gluten has a predominant role in the structure, the use of lipase, by increasing the gluten protein network resistance to cooking also plays a role in reducing the porosity and oil uptake during (noodles) frying (Gulia et al. 2014).

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[1] USA (1970) - US Patent, US 3520702 A "*Method of making dried pasta having a protein network that withstands cooking*")1 available online: http://www.google.com/patents/US3520702

[2] VTT Biotechnology and TNO Nutrition and Food Research Institute (1999) – Second European Symposium on Enzymes in Grain processing - VTT Symposium 207- ESEGP-2 . p. 167 Available online: http://www2.vtt.fi/inf/pdf/symposiums/2000/S207.pdf

Therefore, the benefits of the conversion of the triglycerides (non-polar lipids) with the help of lipase in other cereal-based processes can be summarized as follows:

- Facilitate the handling of the dough (Colakoglu and Özkaya 2012)
- Improve dough stability and strength which results in processing tolerance (Gulia et al. 2014)
- Improved texture after boiling/steaming (Ma et al. 2022)

The process flow of lipase used in other cereal-based processes is presented below:

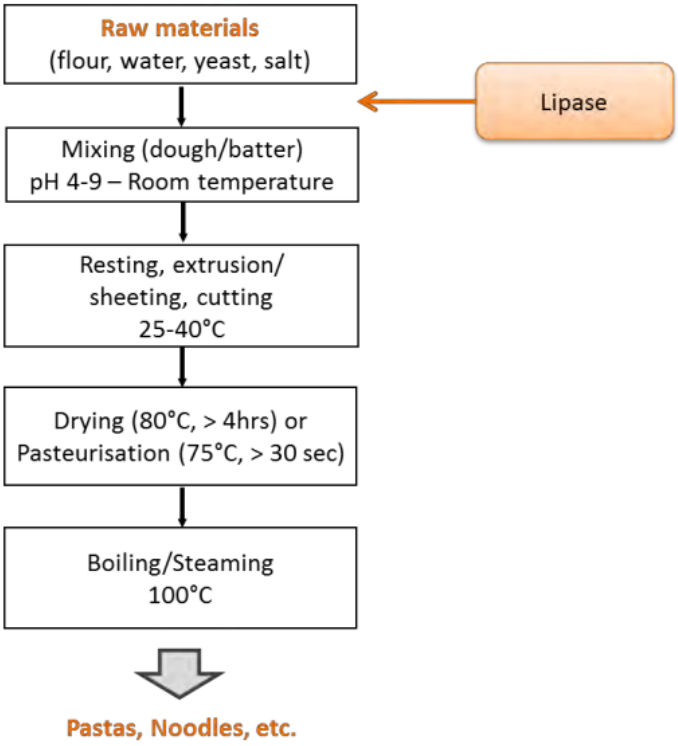

Figure 3: Lipase use in other cereal-based products

Fate of enzyme in food

Like the endogenous lipases present in food raw materials and ingredients, the added lipase does not perform any technological function in the final foods. The reasons why the enzyme does not exert any (unintentional) enzymatic activity in the final food can be due to a combination of various factors, depending on the application and the process conditions used by the individual food producer. These factors include depletion of the substrate, denaturation of the enzyme during processing, lack of water activity, wrong pH, etc. In some cases (e.g., after alcohol distillation), the enzyme may no longer be present in the final food.

In baking, lipases typically perform their technological function during the dough or batter handling. Lipases are denatured by heat during the baking or steaming step.

In cereal-based processes, such as pasta and noodle production, lipases also perform their technological function during dough handling. Afterwards, lipases are denatured by heat during the drying, boiling and/or steaming step.

Consequently, it can be concluded that the lipase does not exert any (unintentional) enzymatic activity in the final foods.

Safety Evaluation

The safety of the lipase produced by the genetically modified *Trichoderma reesei* AR-822 from a toxicological perspective is supported by the historical safety of strain lineage. Toxicological studies were performed on a strain (AR-852) which derives from the same recipient strain as AR-822. Expression constructs are very similar, differing by the expression cassette/enzyme gene of interest. As both production strains are free of any harmful sequences or any potential hazards, the expression cassettes are very similar and are stably integrated into the genome of the strains without any additional mutagenesis cycles thereafter, differences in the genetic modification of AR-822 and AR-852 are not a safety concern. Furthermore, the manufacturing conditions between the two production strains are very similar. The slight changes in pH levels and fermentation medium (food-grade) have been thoroughly assessed. They are considered minor (common industry practice) and do not trigger any additional safety issue.

To add on, enzyme product from AR-822 production strain complies with JECFA specifications for chemical and microbiological purity of food enzymes (Food and Agriculture Organization of the United Nations 2006) which confirms the safety of the production strain AR-822.

With the use of safe strain lineage, we have substantiated the safety of the AR-822 *Trichoderma reesei* production strain via three toxicological studies on the *Trichoderma reesei* AR-852 production strain to demonstrate non-toxigenicity of the strain lineage. The toxicological studies conducted include, a reverse mutation assay using bacteria, a Micronucleus Assay in Bone Marrow Cells of the Rat, and a 90-day repeated dose oral toxicity study in Wister rats. All three toxicological studies showed negative findings demonstrating the AR-852 production strain to be non-mutagenic, to not induce structural and/or numerical chromosomal damage, and to not cause toxigenic effects on the Wister rats tested in the 90-day oral toxicity study.

The product is free of production strain and production strain DNA.

AB Enzymes is in the process of registering the *Trichoderma reesei* AR-822 lipase production strain in other countries such as Brazil (ANVISA), EU (EFSA), and USA (US FDA), and plans to register in Canada (Health Canada). Denmark has approved the use of the enzyme.

Conclusion

To conclude, the use of the food enzyme lipase from *Trichoderma reesei* AR-822 in the production of food is safe based on the following aspects presented in this dossier:

- Safety data and information of the production strain
- Allergenicity and toxin analysis assessment on the amino acid sequence of food enzyme
- TDMI value based on Budget Method

Trichoderma reesei has been used in the food industry for many years. Strains from the *Trichoderma reesei* microorganism are generally recognized as safe and are recognized to produce a variety of enzymes. *Trichoderma reesei* is listed as a permitted producer of enzymes in multiple global food enzyme positive lists, including in Australia. The safety of the lipase produced by the genetically modified *Trichoderma reesei* AR-822 from a toxicological perspective is supported by the historical safety of strain lineage which is provided in the dossier. We have demonstrated that the enzyme batches containing lipase from *Trichoderma reesei* AR-822 meet the following criteria:

- Absence of Antibiotic and Toxic Compounds & Analysis of Purity and Identity Specifications of the Enzyme Preparation
- Absence of Production strain
- No Detection of production strainDNA

Based on the safety evaluation, AB Enzymes GmbH respectfully request the inclusion of **Triacylglycerol Lipase** (EC 3.1.1.3) from *Thermomyces lanuginosus* expressed in a genetically modified strain of *Trichoderma reesei* AR-822 in the table of **Schedule 18—9(3) Permitted processing aids — various purposes (section 1.3.3—11)**.

Publication bibliography

Colakoglu, Abdullah S.; Özkaya, Hazım (2012): Potential use of exogenous lipases for DATEM replacement to modify the rheological and thermal properties of wheat flour dough. In *Journal of Cereal Science* 55 (3), pp. 397–404. DOI: 10.1016/j.jcs.2012.02.001.

Gulia, Neelam; Dhaka, Vandana; Khatkar, B. S. (2014): Instant noodles: processing, quality, and nutritional aspects. In *Critical reviews in food science and nutrition* 54 (10), pp. 1386–1399. DOI: 10.1080/10408398.2011.638227.

Ma, Mengmei; Mu, Taihua; Sun, Hongnan; Zhou, Liang (2022): Evaluation of texture, retrogradation enthalpy, water mobility, and anti-staling effects of enzymes and hydrocolloids in potato steamed bread. In *Food Chemistry* 368, p. 130686. DOI: 10.1016/j.foodchem.2021.130686.

Rodríguez-García, Julia; Sahi, Sarabjit S.; Hernando, Isabel (2014): Functionality of lipase and emulsifiers in low-fat cakes with inulin. In *LWT - Food Science and Technology* 58 (1), pp. 173–182. DOI: 10.1016/j.lwt.2014.02.012.

Sîrbu, Alexandrina; Pâslaru, V. (2005): Influence of Lipase Products on Technological Properties of the Bread Flour. In *Scientifical Researches. Agroalimentary Processes and Technologies* 5 (1), pp. 185–192. Available online at https://www.journal-of-agroalimentary.ro/admin/articole/35317L27_Influence_of_Lipase_Products_on_Technological_Properties_of_the_Bread_Flour.pdf.