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ALST/PNI

Alpha-amylase from *Aspergillus niger*

An application to amend the *Australia New Zealand Food Standards Code* with an alpha-amylase preparation produced by a genetically modified strain of *Aspergillus niger* expressing an alpha-amylase from *Rhizomucor pusillus*

Novozymes A/S
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EXECUTIVE SUMMARY

The present application seeks to amend Schedule 18—Processing aids of the Australia New Zealand Food Standards Code (the Code) to approve an alpha-amylase enzyme preparation produced by Novozymes A/S.

Proposed change to Australia New Zealand Food Standards Code – Schedule 18—Processing aids

Schedule 18—Processing aids is proposed to be amended to include a genetically modified strain of *Aspergillus niger* expressing an alpha-amylase from *Rhizomucor pusillus* as permitted source for alpha-amylase.

The application is applied for assessment by the general procedure.

Description of enzyme preparation

The enzyme is an alpha-amylase (EC 3.2.1.1).

Alpha-amylases catalyse the hydrolysis of 1,4-alpha-D-glucosidic linkages in starch polysaccharides.

The enzyme is produced by submerged fermentation of an *Aspergillus niger* microorganism expressing an alpha-amylase from *Rhizomucor pusillus*.

The alpha-amylase enzyme preparation is available as a liquid preparation complying with the JECFA recommended purity specifications for food-grade enzymes.

The producing microorganism, *Aspergillus niger*, is absent from the commercial enzyme product.

Use of the enzyme

The alpha-amylase preparation is used as a processing aid during starch processing and beverage alcohol (distilling) processes. Generally, alpha-amylases degrade starch into maltose, glucose and dextrins.

- During starch processing to produce syrups the alpha-amylase degrades starch into dextrins.
- In beverage alcohol (distilling) processes the alpha-amylase converts liquified starch into fermentable sugars.

Benefits

The benefits of the action of the alpha-amylase during starch processing are:

- Efficient degradation of starch and production of the suited substrate (dextrins) for further processing and production of syrups.

The benefits of the action of the alpha-amylase in beverage alcohol (distilling) processes:

- Higher ethanol yields
- Fast fermentation
- Efficient production of the suited substrate (dextrins) for saccharification
- Efficient production of fermentable sugars for fermentation

Safety evaluation

The safety of the strain and the enzyme product has been thoroughly assessed:

- The production organism has a long history of safe use as production strain for food grade enzyme preparations and is known not to produce any toxic metabolites.
- The genetic modifications in the production strain are well-characterised and safe and the recombinant DNA is stably integrated into the production organism and unlikely to pose a safety concern.
- The enzyme preparation complies with international specifications ensuring absence of contamination by toxic substances or noxious microorganisms
- Sequence homology assessment to known allergens and toxins shows that oral intake of the alpha-amylase does not pose food allergenic or toxic concern.
- Two mutagenicity studies in vitro showed no evidence of genotoxic potential of the enzyme preparation.
- An oral feeding study in rats for 13-weeks showed that all dose levels were generally well tolerated and no evidence of toxicity.

Furthermore, the safety of the alpha-amylase preparation was confirmed by external expert groups, as follows:

- Denmark: The enzyme preparation was safety assessed resulting in the authorisation of the enzyme product by the Danish Veterinary and Food Administration.
- France: The enzyme is included in the French positive list for processing aids, including food enzymes (The French order of October 19, 2006 on use of processing aids in the manufacture of certain foodstuff), as amended.
- Mexico: Based on a dossier submitted by Novozymes, the Mexican food authorities, COFEPRIS, have approved the enzyme.

Conclusion

Based on the Novozymes safety evaluation (confirmed by the above-mentioned bodies), we respectfully request the inclusion of this enzyme in Schedule 18—Processing aids.

INTRODUCTION

The present dossier describes an alpha-amylase enzyme preparation produced by submerged fermentation of an *Aspergillus niger* microorganism expressing an alpha-amylase from *Rhizomucor pusillus*.

The enzyme is an alpha-amylase (EC 3.2.1.1). The enzyme catalyses the hydrolysis of 1,4-alpha-D-glucosidic linkages in starch polysaccharides

The alpha-amylase enzyme preparation is intended to be used as a processing aid during starch processing and beverage alcohol (distilling) processes.

The following sections describe in detail the construction of the genetically modified *Aspergillus niger* used as the production organism, the production process, the product specification, the application of the enzyme preparation and finally the safety evaluation of the product including the toxicology program, which has been carried out confirming the safety of the product for its intended use.

The documentation has been elaborated according to the Application Handbook from Food Standards Australia New Zealand as of July 1st 2019, applied as relevant for an enzyme application, i.e. outlining the following section:

- Section 3.1.1 – General requirements
- Section 3.3.2 – Processing aids, subsections A, C, D, E, F

NB! When reading this document it should be noticed that in some reports, the alpha-amylase enzyme preparation is described by its commercial name, Amylase NG or by the internal production batch codes PPY31016.

CHAPTER 3.1, GENERAL REQUIREMENTS FOR APPLICATIONS

A Executive Summary

An Executive Summary is provided as a separate copy together with this application.

B Applicant details

(a) Applicant's name/s

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(f) Nature of applicant's business
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C Purpose of the application

This application is submitted to provide for amendment of the Australia New Zealand Food Standards Code, Schedule 18—Processing aids to include a genetically modified strain of *Aspergillus niger* as permitted source for alpha-amylase.

D Justification for the application

The need for the proposed change

Schedule 18—Processing aids contains a list of permitted enzymes of microbial origin. There are a number of approved alpha-amylase EC 3.2.1.1 from different sources, including *Aspergillus niger*. However, Schedule 18—Processing aids does not contain an alpha-amylase EC 3.2.1.1 from *Aspergillus niger* containing the gene for alpha-amylase from *Rhizomucor pusillus*.

Aspergillus niger is an approved host and production strain for a number of enzymes in Schedule 18—Processing aids, e.g. a wide range of enzymes that can be used in starch processing such as alpha-amylase, alpha-arabinofuranosidase, cellulase, endo-1,4-beta-xylanase, endo-arabinase, alpha-galactosidase, beta-galactosidase, beta-glucanase, glucoamylase, alpha-glucosidase, beta-glucosidase, hemicellulase multicomponent enzyme.

The advantages of the proposed change over the status quo

The alpha-amylase preparation is used as a processing aid during starch processing, and beverage alcohol (distilling) processes. Alpha-amylases hydrolyse starch molecules randomly releasing maltose, glucose and dextrins for further processing to a wide range of products, such as syrup and distilled alcohol.

The benefits of the action of the alpha-amylase during starch processing are:

- Efficient degradation of starch and production of the suited substrate (dextrins) for further processing and production of syrups.

The benefits of the action of the alpha-amylase in beverage alcohol (distilling) processes:

- Higher ethanol yields
- Fast fermentation
- Efficient production of the suited substrate (dextrins) for saccharification
- Efficient production of fermentable sugars for fermentation

The benefits, which are described above, are not exclusively obtainable by means of enzyme treatment but can be achieved without the use of enzymes, or with a reduced use of enzymes, through e.g. modified production processes or recipe changes that may be more expensive or less environmentally friendly.

As a response to international customer interests, registration activities have been done globally, e.g. the alpha-amylase enzyme preparation has been approved in Denmark, France, and Mexico for the described applications.

D.1 Regulatory impact information

D.1.1 Costs and benefits of the application

The application is not likely to place costs or regulatory restrictions on industry or consumers. Inclusion of the alpha-amylase enzyme in Schedule 18—Processing aids will provide the food and beverage industry with the opportunity to improve the yield of fermentable sugars in starch processing and distilling under environmentally friendly and cost efficient production conditions. For government, the burden is limited to necessary activities for a variation of Schedule 18—Processing aids.

D.1.2 Impact on international trade

The application is not likely to cause impact on international trade.

E Information to support the application

E.1 Data requirements

No public health and safety issues related to the proposed change are foreseen. As outlined in sections 3.3.2 C, D, E, F, the alpha-amylase is produced by submerged fermentation of a genetically modified *Aspergillus niger* strain.

The safety of the strain and the enzyme product has been thoroughly assessed:

- The production organism has a long history of safe use as production strain for food grade enzyme preparations and is known not to produce any toxic metabolites.
- The genetic modifications in the production strain are well-characterised and safe and the recombinant DNA is stably integrated into the production organism and unlikely to pose a safety concern.
- The enzyme preparation complies with international specifications ensuring absence of contamination by toxic substances or noxious microorganisms
- Sequence homology assessment to known allergens and toxins shows that oral intake of the alpha-amylase does not pose food allergenic or toxic concern.
- Two mutagenicity studies *in vitro* showed no evidence of genotoxic potential of the enzyme preparation.
- An oral feeding study in rats for 13-weeks showed that all dose levels were generally well tolerated and no evidence of toxicity.

F Assessment procedure

Because the application is for a new source organism for an existing enzyme in the Code, it is considered appropriate that the assessment procedure is characterised as “General Procedure, Level 1”.

G Confidential commercial information (CCI)

Detailed information on the construction and characteristics of the genetically modified production strain is provided in Appendix 6. A summary of this information is given in section 3.3.2 E. The formal request for treatment of selected parts of Appendix 6 as confidential commercial information (CCI) is included as Appendix 1.1.

H Other confidential information

Apart from the selected parts of Appendix 6 identified as confidential commercial information (CCI), no other information is requested to be treated as confidential.

I Exclusive capturable commercial benefit (ECCB)

This application is not expected to confer an Exclusive Capturable Commercial Benefit.

J International and other national standards

J.1 International Standards

Use of enzymes as processing aids for food production is not restricted by any Codex Alimentarius Commission (Codex) Standards.

J.2 Other national standards or regulations

With few exceptions on national, commodity standards, use of enzymes as processing aids for food production is in general not restricted by standards or regulations in other countries.

K Statutory declaration

The Statutory Declaration is provided as a separate document together with this submission.

L Checklist

This application concerns an enzyme product intended to be used as a processing aid. Therefore, the relevant documentation according to the Application Handbook from Food Standards Australia New Zealand as of July 1st 2019, are the following sections:

- Section 3.1.1 – General requirements

- Section 3.3.2 – Processing aids, subsections A, C, D, E, F

Accordingly, the checklist for General requirements as well as the Processing aids part of the checklist for applications for substances added to food was used and is included as Appendix 1.2 and 1.3.

CHAPTER 3.3, GUIDELINES FOR APPLICATIONS FOR SUBSTANCES ADDED TO FOOD

3.3.2 PROCESSING AIDS

The alpha-amylase enzyme preparation described in this application is representative of the commercial food enzyme product for which approval is sought.

A Technical information on the processing aid

A.1 Information on the type of processing aid

The alpha-amylase enzyme preparation belongs to the category of processing aids described in Schedule 18—Processing aids.

The alpha-amylase enzyme preparation is to be used in the food industry as a processing aid during the processing of raw materials containing starch. Alpha-amylase converts starch to maltose, glucose and dextrin.

The alpha-amylase enzyme preparation is used in, but not limited to, the following food manufacturing processes:

- During starch processing to produce syrups the alpha-amylase degrades starch into dextrins.
- In beverage alcohol (distilling) processes the alpha-amylase converts liquified starch into fermentable sugars.

The recommended and highest dosage in all processes listed above is up to 200 FAU(F) per kg starch dry matter.

A.2 Information on the identity of the processing aid

A.2.1 Enzyme

Generic name	Alpha-amylase
IUBMC nomenclature:	4-α-D-glucan glucanohydrolase
IUBMC No.:	EC 3.2.1.1
Cas No.:	9000-90-2

A.2.2 Enzyme preparation

The enzyme concentrate is formulated into a final enzyme preparation. The enzyme concentrate may be intended for a single enzyme preparation or a blend with other food enzymes and formulated as a liquid product or a granulate depending on the characteristics of the intended food process in which it will be used.

The typical compositions of the enzyme concentrate is shown below:

	Typical composition of enzyme concentrate
Enzyme solids (TOS[1])	approx. 35.0 %
Sucrose/glucose	approx. 8.3 %
Sodium benzoate	approx. 0.4 %
Potassium sorbate	approx. 0.1 %
Sodium metabisulfite	approx. 0.2 %
Water	approx. 56.0 %

The enzyme concentrate is standardised in alpha-amylase units to an activity of 150 FAU(F)/g. The Novozymes method used to determine the FAU(F) activity is enclosed in Appendix 3.1.

Alpha-amylase converts ethylidene-G7-p-nitrophenyl-maltoheptaoside in presence of an alpha-glucosidase to glucose and the yellow-coloured p-nitrophenol which can be followed and quantified at 405 nm. The colour development is monitored, so the change in absorption per time unit can be calculated. The increase in absorption is proportional to the enzyme activity.

A.2.3 Host organism

The host strain is a modified (protease deficient) *Aspergillus niger* strain (BO-1) derived from a natural isolate of *Aspergillus niger* C40-1. The BO-1 strain lineage has been used by Novozymes for more than 30 years and has given rise to a number of food enzyme production strains, which are used for production of previously evaluated and regulatory approved food enzymes. The taxonomic classification of the strain is as follows:

Name:	*Aspergillus niger*
Class:	Eurotiomycetes
Order:	Eurotiales
Genus:	*Aspergillus*
Species:	*niger*

For a more detailed description of the host organism and the genetic modifications, please see section 3.3.2 E.

A.2.4 Donor organism

The donor for the alpha-amylase gene is *Rhizomucor pusillus*.

For a more detailed description of the donor and the donor gene, please see section 3.3.2 E.

A.3 Information on the chemical and physical properties of the processing aid

The enzyme is an alpha-amylase (EC 3.2.1.1). Alpha-amylases catalyse the hydrolysis of 1,4-alpha-D-glucosidic linkages in starch polysaccharides.

[1] TOS = Total Organic Solids, defined as: 100 % - water - ash - diluents

The enzyme preparation is available as a liquid product stabilised with sucrose and glucose.

The food enzyme object of the present dossier is not added to final foodstuffs but used as a processing aid during food manufacturing.

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.

A.4 Manufacturing process

The manufacturing process is composed of a fermentation process, a purification process, a formulation process and finally a quality control of the finished product, as outlined by Aunstrup et al. (1979)[1] . This section describes the processes used in manufacturing of the alpha-amylase enzyme product.

The enzyme preparation is manufactured in accordance with current Good Manufacturing Practices, Food. The quality management system used in the manufacturing process complies with ISO 9001:2015 (Appendix 4).

The raw materials are Food Grade Quality and have been subjected to appropriate analysis to ensure their conformity with the specifications.

A.4.1 Fermentation

The alpha-amylase is produced by submerged fed-batch pure culture fermentation of the genetically modified strain of *Aspergillus niger*, described in section 3.3.2 E.

A.4.1.1 Raw materials for fermentation

The production strain is grown in a medium consisting of compounds providing an adequate supply of carbon and nitrogen plus minerals and vitamins necessary for growth. The choice of raw materials used in the fermentation process (the feed, the seed fermenter, the main fermenter and dosing) is listed below.

- Potable water
- Carbohydrates (e.g. corn starch, glucose syrup, sucrose)
- Vegetable protein (e.g. soy bean meal)
- Ammonia
- Salts (e.g. magnesium sulphate, potassium hydroxide, sulphuric acid, ammonia)
- Trace metals (e.g. $NiCl_2$, $MnSO_4$, $FeSO_4$, $CuSO_4$, $ZnSO_4$)
- Alkali and acid for pH adjustments (e.g. citric acid, phosphoric acid, sodium hydroxide, ammonia)
- Antifoaming agents (e.g. polypropylene glycol, polyalkoxyether)

A.4.1.2 Hygienic precautions

All equipment is designed and constructed to prevent contamination by foreign microorganisms.

All valves and connections not in use for the fermentation are sealed by steam at more than 120 °C.

After sterilization a positive pressure of more than 0.2 atmosphere is maintained in the fermentation tank.

The air used for aeration is sterilised by passing a sterile filter.

The inside of each fermentation tank is cleaned between fermentations by means of a high-pressure water jet and inspected after the cleaning procedures have been completed.

A.4.1.3 Preparation of the inoculum

The inoculum flask containing the prepared medium is autoclaved and checked. Only approved flasks are used for inoculation.

The stock culture suspension is injected aseptically into the inoculum flask and spread onto the medium in the flask. Once growth has taken place in the inoculum flask (typically after a few days at 30°C), the following operations are performed:

- Strain identity and traceability: ampoule number is registered
- Microbial purity: a sample from the inoculum flask is controlled microscopically for absence of microbial contaminants.

When sufficient amount of biomass is obtained and when the microbiological analyses are approved, the inoculum flask can be used for inoculating the seed fermenter.

A.4.1.4 The seed fermentation

The raw materials for the fermentation medium are mixed with water in a mixing tank. The medium is transferred to the seed fermenter and heat sterilised (e.g. 120 °C / 60 min).

The seed fermentation tank is inoculated by transferring aseptically a suspension of cells from the inoculum flask.

The seed fermentation is run aerobically (sterile airflow), under agitation. The overpressure is kept above 0.2 atmosphere at all times, to prevent contamination.

Once a sufficient amount of biomass has developed, microbiological analyses are performed to ensure absence of contamination. The seed fermentation can then be transferred to the main fermentation tank.

A.4.1.5 The main fermentation

The raw materials for the medium are mixed with water in a mixing tank. The medium is transferred to the main fermenter and heat sterilised (e.g. 120°C / 60 min). If necessary, the pH is adjusted after sterilization, with sterile pH adjustment solutions.

The fermentation in the main tank is run as normal submerged fed-batch fermentation.

The main fermentation is run aerobically (sterile airflow), under vigorous agitation. The overpressure is kept above 0.2 atmosphere at all times, to prevent contamination. The fermentation is run at a well-defined temperature.

Fresh medium is added aseptically when the pH increases above its set point, and the dissolved oxygen concentration rises. The feed rate is adjusted so that there is no accumulation of carbohydrates.

Other parameters are measured at regular intervals

- Refractive index
- Enzyme productivity
- Residual glucose
- Residual ammonia.

Samples are also taken at regular intervals to check absence of microbial contamination.

A.4.2 Recovery

The recovery process is a multi-step operation designed to separate the enzyme from the microbial biomass and partially purify, concentrate, and stabilize the food enzyme.

The steps of this process involve a series of typical unit operations:

- Pre-treatment
- Primary separation
- Filtration
- Concentration
- Preservation and stabilization
- Filtration

A.4.2.1 Raw materials for recovery

The raw materials typically used in the recovery process are as follows:

- Potable water
- Filter aids or pre-coats (e.g. diatomaceous earth, perlite
- Acids and bases for pH adjustment (e.g. phosphoric acid, sodium hydroxide)
- Flocculants (e.g. polyacrylamide, polyaluminium chloride)
- Stabilisation (e.g. sucrose, potassium sorbate, sodium benzoate)
- Antifoam (if necessary, polymer of fatty acids and alkanehydrocarbon)

A.4.2.2 Pre-treatment

To facilitate the separation, flocculants are used in a pH-controlled process.

A.4.2.3 Primary separation

The cell mass and other solids are separated from the broth by well-established techniques such as pre-coat vacuum drum filtration or centrifugation. The precoat used in the filter and the filter aid used in the process is diatomaceous earth (diatomite or perlite).

The primary separation is performed at well-defined pH and temperature range.

A.4.2.4 Filtration

For removal of residual cells of the production strain and as a general precaution against microbial degradation, filtration on dedicated germ filtration media is applied. Pre-filtration is included when needed.

The filtrations are performed at well-defined pH and temperature intervals, and result in an enzyme concentrate solution free of the production strain and insoluble substrate components from the fermentation.

A.4.2.5 Concentration

Ultrafiltration and/or evaporation are applied for concentration and further purification. The ultrafiltration is applied to fractionate high molecular weight components (enzymes) from low molecular weight components and is used to increase the activity/dry matter ratio. Evaporation is used to increase the activity while maintaining the activity/dry matter ratio.

The pH and temperature are controlled during the concentration step, which is performed until the desired activity and activity/dry matter ratio has been obtained.

A.4.2.6 Preservation and stabilization

For enzymatic, physical and microbial stabilization glycerol is added to the enzyme concentrate. pH is adjusted by acetic acid or sodium hydroxide.

A.4.2.7 Final filtration

A polish filtration is performed to remove any precipitations followed by a final germ filtration. The enzyme concentrate is stored at 5-10 °C.

A.4.2.8 Process control

Apart from the process controls performed during the various fermentation steps and described above, the following microbial controls are also performed.

Samples are withdrawn from both the seed fermenter and the main fermenter:

- before inoculation
- at regular interval during cultivation
- before transfer/harvest

The samples during all steps are examined by:

- microscopy
- plating culture broth on a nutrient agar and incubating for 24-48 hours.

Growth characteristics are observed macroscopically and microscopically.

During the microbiological control steps, the number of foreign microorganisms should be insignificant. The fermentation parameters, i.e. enzyme activity, temperature and oxygen as well as pH are also monitored closely. A deviation from the normal course of the fermentation may signal a contamination.

If a significant contamination develops, the fermentation is terminated. The fermentation is regarded as “significantly contaminated” if two independent samples show presence of contaminating organisms after growth on nutrient agar.

Any contaminated fermentation is rejected for enzyme preparations to be used in a food grade application.

A.5 Specification for identity and purity

The alpha-amylase enzyme product complies with the purity criteria recommended for Enzyme Preparations in Food, Food Chemicals Codex, 11th edition, 2018.

In addition to this, the alpha-amylase enzyme product also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications, available online at: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/.

Analytical data for an unstandardised, representative batch of the alpha-amylase enzyme product is shown in Table 1. These data show compliance with the purity criteria of the specification.

Table 1 Analytical data for the enzyme product batch PPY31016

Control parameter	Unit	Specification	Batch PPY31016
Alpha-amylase activity	FAU(F)/g		53.3
Heavy Metals[a]	ppm	Max 30	3.5
Pb	ppm	Max 5	ND[b] (DL[c] < 0.5)
As	ppm	Max 3	ND (DL < 0.1)
Cd	ppm	Max 0.5	ND (DL < 0.05)
Hg	ppm	Max 0.5	ND (DL < 0.03)
Total viable count	per g	Not more than 50000	600
Total coliforms	per g	Not more than 30	<10
Enteropathogenic *E. coli*	per 25 g	Not detected	ND

Control parameter	Unit	Specification	Batch PPY31016
Salmonella	per 25 g	Not detected	ND
Antibiotic activity		Not detected	ND
Ochratoxin A	ppm		ND (DL < 0.0003)
Fumonisin B2	ppm		ND (DL < 0.0005)
Production strain	per g	Not detected	ND

a) Heavy metals = Sum of Ag, As, Bi, Cd, Cu, Hg, Mo, Ni, Pb, Sb, Sn
b) ND = not detected
c) DL = detection limit

The methods of analysis used to determine compliance with the specifications are enclosed (Appendix 3).

The alpha-amylase enzyme preparation is available as a liquid enzyme concentrate. The concentrate is standardised in alpha-amylase units (FAU(F)/g, Appendix 3.1). The preparation does not contain known food allergens.

A.6 Analytical method for detection

The alpha-amylase enzyme preparation is to be used in the food industry as a processing aid. This information is not required in the case of an enzymatic processing aid.

B Information related to the safety of a chemical processing aid

Not applicable – this application does not concern a chemical processing aid.

C Information related to the safety of an enzyme processing aid

C.1 General information on the use of the enzyme as a food processing aid in other countries

The enzyme is used as processing aid during processing of starch-containing raw materials in a range of countries, where there are no restrictions of the use of enzyme processing aids or where the enzyme is covered by country positive list or specific approval.

The safety of the alpha-amylase preparation has been evaluated and confirmed by external expert groups, as follows:

- Denmark: The enzyme preparation was safety assessed resulting in the authorisation of the enzyme product by the Danish Veterinary and Food Administration.
- France: The enzyme is included in the French positive list for processing aids, including food enzymes (The French order of October 19, 2006 on use of processing aids in the manufacture of certain foodstuff), as amended.

- Mexico: Based on a dossier submitted by Novozymes, the Mexican food authorities, COFEPRIS, have approved the enzyme.

C.2 Information on the potential toxicity of the enzyme processing aid

(a) Information on the enzyme's prior history of human consumption and/or its similarity to proteins with a history of safe human consumption

A wide variety of enzymes are used in food processing. Enzymes, including alpha-amylase, have a long history of use in food (Pariza and Foster, 1983[2]; Pariza and Johnson, 2001[3]).

Since the 1960s alpha-amylases have been used extensively in various industrial food applications for the hydrolysis of starch (Reed, 1966[4,5,6]; Pence, 1953[7]). The first conditions of the use of fungal alpha-amylase in starch hydrolysis have been described in the patents of Takamine (1894[8], 1896[9]). Some of the technical aspects of the use of alpha-amylase in food production such as sweetener production, alcohol distillation, baking etc. are described in the book 'Enzyme arbeiten für uns' (Uhlig, 1991[10]). Microbial amylases have to a large extent replaced chemical hydrolysis in the starch processing industry (Gupta et al, 2003[11]; Van der Maarel et al, 2002[12]). Alpha-amylase enzyme preparations from various sources are widely authorised in, e.g. Australia and New Zealand, Brazil, Canada, China, Denmark, France, Japan, Mexico.

(b) Information on any significant similarity between the amino acid sequence of the enzyme and that of known protein toxins

A sequence homology assessment of the alpha-amylase enzyme to known toxins and allergens was conducted. The amino acid sequence of the alpha-amylase provided in Appendix 6.3 was used as input for the search. No homologies to toxins or allergens were found. The complete search report is enclosed in Appendix 5.1.

Furthermore, safety studies as described below were performed on a representative batch (PPY31016) that was produced according to the description given in section 3.3.2 A.4, omitting stabilization and standardization. A summary of the safety studies is enclosed in Appendix 5.2.

The following studies were performed:

- Ames Test. Test for mutagenic activity (Appendix 5.3)
- *In vitro* micronucleus test (Appendix 5.4)
- Subchronic (13 week) oral toxicity study in rats (Appendix 5.5)

The main conclusions of the safety studies can be summarised as follows:

- Alpha-amylase, PPY31016 did not induce gene mutations in bacteria either in the presence or absence of metabolic activation (S-9) when tested under the conditions employed in this study.
- Alpha-amylase, PPY31016 did not induce micronuclei in cultured human peripheral blood lymphocytes following treatment in the presence or absence of an aroclor induced rat liver metabolic activation system (S-9).

- Oral administration of batch PPY31016 to Sprague-Dawley rats at doses up to 100% of the tox test batch (1220 mg TOS/kg bw/day for 13 weeks was well-tolerated and did not cause any adverse change. The NOAEL was considered to be 100% of the tox test batch (equivalent to 1220 mg TOS/kg bw/day).

Based on the present toxicity data it can be concluded that the alpha-amylase enzyme preparation, represented by batch PPY31016 exhibits no toxicological effects under the experimental conditions described.

C.3 Information on the potential allergenicity of the enzyme processing aid

(a) Information of the source of the enzyme processing aid

The alpha-amylase enzyme is produced by an *Aspergillus niger* microorganism expressing the alpha-amylase from *Rhizomucor pusillus*. *Aspergillus niger* is ubiquitous in the environment and in general considered as a non-pathogenic fungus (see Section 3.3.2 D).

(b) Analysis of similarity between the amino acid sequence of the enzyme and that of known allergens

Enzymes have a long history of safe use in food, with no indication of adverse effects or reactions. Moreover a wide variety of enzyme classes (and structures) are naturally present in food.

The allergenicity potential of enzymes was studied by Bindslev-Jensen et al (2006)[13] and reported in the publication: "Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry". The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.

Additionally, food enzyme are used in small amounts during food processing resulting in very small amounts of the enzyme protein in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman et al, 2008)[14].

A sequence homology assessment of the alpha-amylase enzyme to known toxins and allergens was conducted (Appendix 5.1). The amino acid sequence of the alpha-amylase provided in Appendix 6.3 was used as input for the search. The alpha-amylase was compared to allergens from the FARRP allergen protein database (http://www.allergenonline.org)[15] as well as the World Health Organisation and International Union or Immunological Societies (SHO/IUIS) Allergen Nomenclature Sub-committee (http://www.allergen.org)[16] using the recommended allergen method by EFSA.

Allergen risk assessment analysis of the alpha-amylase was performed according to the EFSA scientific opinion using *allergen online* and *allergen.org* databases. The analyses of the alpha-amylase's sequence identified homology to Sch c 1 and Asp o 21, two known allergens, above the threshold of 35 % across an 80 amino acid window (Appendix 5).

Glucoamylase Sch c 1 of *Schizophyllum commune* was revealed to have up to 55.2 % identity with the alpha-amylase produced by *Aspergillus niger*, while alpha-amylase Asp o 21 of *Aspergillus oryzae* has up to 60.3 % identity across an 80 amino acids window using the *allergen.org* database. Both are described as occupational allergens (by inhalation) and therefore should not be of concern to oral intake.

This is backed up by two studies using the generally recognised guidelines for food allergy diagnosis (skin prick test, specific serum IgE and DBPCFC). One study included 18 patients with occupational (inhalation) allergy to fungal alpha-amylase and screening of sera from 1000 persons from the general population for IgE sensitisation to alpha-amylase (Poulsen, 2004[17]). The other study (Bindslev-Jensen et al, 2006[13] as also described above) included 400 patients with diagnosed allergy to one or more of: inhalation allergens, food allergens, bee or wasp allergens. Both studies concluded that no cases of IgE-mediated food allergy to commercial enzymes could be found. It should be further noted that five of the tested enzymes were alpha-amylases. There were no indications of cross-reactivity between the tested enzymes used in food and the main known allergens represented by the patients included in the study by Bindslev-Jensen et al, 2006[13].

On the basis of the available evidence it is concluded that oral intake of the alpha-amylase is not anticipated to pose any food allergenic concern.

C.4 Safety assessment reports prepared by international agencies or other national government agencies, if available

The certificate of approval of the alpha-amylase enzyme preparation by the Danish authorities following their safety evaluation in accordance with the principles laid down in the Guidelines for the presentation of data on food enzymes, “cf. Reports of the Scientific Committee of Food, 27th Series, EUR 14181, 1992” is enclosed as Appendix 2.

D Additional information related to the safety of an enzyme processing aid derived from a microorganism

D.1 Information on the source microorganism

The alpha-amylase enzyme is produced by an *Aspergillus niger* microorganism expressing the alpha-amylase from *Rhizomucor pusillus*. The host strain is a modified (protease deficient) *Aspergillus niger* strain (BO-1) derived from a natural isolate of *Aspergillus niger* C40-1. The BO-1 strain lineage has been used by Novozymes for more than 30 years and has given rise to a number of food enzyme production strains, which are used for production of previously evaluated and regulatory approved food enzymes.

The alpha-amylase production strain is a non-pathogenic, non-toxigenic, genetically-modified *Aspergillus niger* strain. The production strain is marker-free, and it does not produce secondary metabolites of toxicological concern to humans as explained in Section E 1.3, Section A.5 and Appendix 6.1.

D.2 Information on the pathogenicity and toxicity of the source microorganism

Aspergillus niger is ubiquitous in the environment and in general considered as a non-pathogenic fungus.

Aspergillus niger is classified as a group 1 microorganism according to EU Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work. A group 1 microorganism means one that is unlikely to cause human disease.

Aspergillus niger as a species has been used safely for the production of food ingredients (e.g. citric acid) and food enzymes world-wide for decades.

Schuster et al. (2002)[18] reviewed the safety of *Aspergillus niger* and describe it as having a very long history of safe industrial use, being widely distributed in nature, and being commonly used for production of food enzymes and citric acid.

Aspergillus niger has been used in the industry since 1919, for instance for the production of citric acid, which could be an ingredient of foods such as soft drinks, fruit juices and jams. The US Food and Drug Administration (FDA) has listed *Aspergillus niger* as a source of citric acid (21 CFR §173.280).

The JECFA (Joint FAO/WHO Expert Committee on Food Additives) has evaluated enzyme preparations derived from *Aspergillus niger*. This body of experts determined that enzymes from this source do not constitute a toxicological hazard (WHO, 1990)[19].

Carbohydrase, pectinase, protease, glucose oxidase, catalase, lipase and lactase enzyme preparations from *Aspergillus niger* are included in the GRAS petition 3G0016 (filed April 12th, 1973) that FDA on request from the Enzyme Technical Association (ETA) converted into separate GRAS Notices11 (GRN 89, 111, 132). Based on the information provided by ETA, as well as the information in GRP 3G0016 and other information available to FDA, the agency did not question the conclusion that enzyme preparations from *Aspergillus niger* are GRAS under the intended conditions of use. Analogous conclusions were drawn in GRAS Notices GRN 158, 183, 214, 296, 345, 402, 428, 510, 651, 657, 699, and 703 which all describe food enzymes produced by *Aspergillus niger* strains.

Overall, it can be concluded that *Aspergillus niger* is widely accepted as a non-pathogenic organism and has a long history of safe use in food and food enzyme production.

D.3 Information on the genetic stability of the source organism

The inserted recombinant DNA is genetically stable during fermentation, as the inserted DNA is integrated into the chromosome.

The genetic stability of the production strain was tested at large-scale fermentation. The strain stability during fermentation was analysed by Southern blotting. No instability of the strain was observed.

For a more detailed description of the strain construction and characteristics, please see section 3.3.2 E.

E Additional information related to the safety of an enzyme processing aid derived from a genetically-modified microorganism

E.1 Information on the methods used in the genetic modification of the source organism

This section contains summarised information on the modifications of the host strain, on the content and nature of the introduced DNA and on the construction of the final production strain, as well as the stability of the inserted gene. The detailed information is provided in the confidential Appendix 6.

E.1.1 Host organism

The host strain is a modified (protease deficient) *Aspergillus niger* strain (BO-1) derived from a natural isolate of *Aspergillus niger* C40-1. The BO-1 strain lineage has been used by Novozymes for more than 30 years and has given rise to a number of food enzyme production strains, which are used for production of previously evaluated and regulatory approved food enzymes. The taxonomic classification of the strain is as follows:

Name:	*Aspergillus niger*
Class:	Eurotiomycetes
Order:	Eurotiales
Genus:	*Aspergillus*
Species:	*niger*

The classification of *Aspergillus niger* BO-1 was confirmed by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

The recipient strain used in the construction of the *Aspergillus niger* production strain, was derived from the C40-1 parental strain through a combination of classical mutagenesis/selection and GM-steps. These steps included the inactivation of proteins involved in protein glycosylation and other strain improvements for product purity, stability and safety.

E.1.2 Introduced DNA

The vectors pHUda634 and pHUda667, used to transform the *Aspergillus niger* recipient strain are based on the well-known *Escherichia coli* standard vector pUC19 (Vieira and Messing, 1987)[20]. No elements of these vectors are left in the production strain. Both vectors contain an expression cassette, *amyAM782*, consisting of a hybrid *Aspergillus* promoter with promoter elements from *Aspergillus niger* and *Aspergillus nidulans*, the coding sequences for alpha-amylase from *Rhizomucor pusillus* and a starch binding domain (SBD) from *Aspergillus niger,* and an *Aspergillus niger* terminator. Furthermore, the vectors contain expression cassettes to introduce two marker genes for selection of successful transformants during the construction of the production strain (*hemA* and *amdS*).

E.1.3 Construction of the Recombinant Microorganism

The *Aspergillus niger* production strain was constructed from recipient strain through the following steps:

1. Plasmid pHUda634 was integrated into random loci in the recipient strain. This lead to the integration of the *amyAM782* and *hemA* expression cassettes.
2. The resulting strain was transformed using plasmid pHUda667, leading to the random integration of *amyAM782* and *amdS* expression cassettes, resulting in the final production strain.

E.1.4 Antibiotic Resistance Gene

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications. The absence of these genes in the production strain was verified by Southern blot analysis using the relevant antibiotic resistance gene probes.

E.1.5 Stability of the Introduced Genetic Sequences

The presence of the introduced DNA sequences was also determined by Southern hybridization to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism. The transforming DNA is stably integrated into the *Aspergillus niger* chromosome and, as such, is poorly mobilised for genetic transfer to other organisms and is mitotically stable.

F Information related to the dietary exposure to the processing aid

F.1 A list of foods or food groups likely to contain the processing aid or its metabolites

The alpha-amylase preparation is used as a processing aid during starch processing, and beverage alcohol (distilling) processes during the pre-saccharification of liquified starch. Alpha-amylases hydrolyse starch molecules randomly releasing maltose, glucose and dextrins for further processing to a wide range of products, such as syrup and distilled alcohol.

F.2 The levels of residues of the processing aid or its metabolites for each food or food group

The alpha-amylase enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following GMP.

The enzyme is used during four methods for processing raw materials containing starch.

- During starch processing to produce syrups the alpha-amylase degrades starch into dextrins.
- In beverage alcohol (distilling) processes the alpha-amylase converts liquified starch into fermentable sugars.

Use level

The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following GMP.

The conditions of use of the alpha-amylase preparation during food processing do not only depend on the type of application, but also on the food production process of each individual food manufacturer. In order to ensure optimal effectiveness of the enzyme at an acceptable economic cost the dosage, reaction time, process conditions and processing steps are adjusted.

The highest dosage given for solid food is 200 FAU(F) per kg starch dry matter. This corresponds to 70 mg of alpha-amylase enzyme preparation per kg starch dry matter equivalent to 434 mg TOS per kg starch dry matter.

The highest dosage given for liquids (excluding distilled beverage spirits *vide supra*) is 200 FAU(F) per kg starch dry matter. This corresponds to 70 mg of alpha-amylase enzyme concentrate per kg starch dry matter equivalent to 434 mg TOS per kg starch dry matter.

Enzyme residues in the Final Food

The alpha-amylase preparation is used in processing of raw materials containing starch for the hydrolysis of starch to maltose, glucose, and dextrins. The enzyme is used during starch processing to produce highly purified syrups and in beverage alcohol (distilling) processes. In both processes the amounts of alpha-amylase and TOS in the final food are negligible. The European Food Safety Agency came to the same conclusion[21].

F.2.1 Estimates of human consumption

As described above the amounts of alpha-amylase and TOS in the final food is negligible. Consequently, no dietary exposure is calculated.

F.2.2 Safety Margin Calculation

As described above the amounts of alpha-amylase and TOS in the final food is negligible. Consequently, calculation of the safety margin is not feasible.

F.3 For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption

Not relevant.

F.4 The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

It is assumed that all raw materials containing starch are processed using the alpha-amylase object of this dossier as a processing aid at the highest recommended dosage.

F.5 Information relating to the levels of residues in foods in other countries

The enzyme is used during starch processing to produce highly purified syrups and in beverage alcohol (distilling) processes. In both processes the amounts of alpha-amylase and TOS in the final food are negligible. The European Food Safety Agency came to the same conclusion[21].

F.6 For foods where consumption has changed in recent years, information on likely current food consumption

No significant changes in recent years are observed.

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