DSM

APPLICATION FOR THE AMENDMENT OF THE AUSTRALIA / NEW ZEALAND FOOD STANDARDS CODE STANDARD 1.3.3 WITH AMYLOMALTASE FROM A GENETICALLY MODIFIED STRAIN OF *BACILLUS AMYLOLIQUEFACIENS*

EXECUTIVE SUMMARY

DSM Food Specialties (“DSM”) manufactures amylomaltase, which is produced by submerged fermentation of a selected, pure culture of a *Bacillus amyloliquefaciens* expressing the amylomaltase gene from *Thermus thermophilus*. DSM produces the amylomaltase preparations in liquid form. It is standardized with glycerol. Trade name is Meltamase™.

The amylomaltase will be used in the processing of Etenia™, a gelatine, starch and casein replacer. As such, applications in foodstuffs can be yoghurt and drink yoghurt.

In this dossier the application of the amylomaltase enzyme produced with a genetically modified strain of Bacillus *amyloliquefaciens* is described. The dossier has been written in the format of the Food Standards Australia New Zealand, Application Handbook, and issue 1 July 2010. The application is related to the Standards for food production and is addressed to the information requirements of Section 3.1 (General requirements) and Sub-section 3.3.2 (Processing aids).

Purpose of this application is to amend Standard 1.3.3. Processing Aids, section 17 by the addition of amylomaltase (EC 2.4.1.25) produced by a *Bacillus amyloliquefaciens* strain containing the gene for amylomaltase from *Thermus thermophilus.*

The amendment is supported in this dossier by:

- Section I, containing general information
- Section II, containing specific information on amylomaltase as a processing aid, consisting of:
    - Section A with information on the purpose, justification and support for the application
    - Section B with technical information on the processing aid
    - Section D with information on the safety of the processing aid including the results of toxicological studies and a conclusion and margin of safety; the conclusion is that the processing was proved to be safe by studies and tests.
    - Section E with additional information on the microbiological issues including safety and stability aspects of the source micro-organism
    - Section F with additional information of the safety aspects concerning the genetic modification of the micro-organism
    - Section G with information of the dietary exposure to the processing aid
    - A list of relevant annexes and references in respectively the Sections H and I
- Section III, containing a checklist according to the Application Handbook, Section 3.1.11 (General requirements).
- Section IV, containing the confidential parts of the dossier

Remarks:

1. The electronic version of this dossier has been divided over the following files:
    - Registration dossier amylomaltase
    - 24 Annexes, each containing 1 or more files
    - 16 References

I GENERAL INTRODUCTION

1 Applicant details

DSM Food Specialties

PO Box 1
2600 MA Delft
The Netherlands

Nature applicant

DSM Food Specialties develops, produces and sells a broad spectrum of ingredients for the food industry.

Manufacturer

DSM Food Specialties

15 Rue des Comtesses
PO Box 239
59472 Seclin Cédex
France
Tel: 33 320964545
Fax: 33 320964500

Person responsible for the dossier

M.E.M. Kuilman
Regulatory Affairs
DSM Nutritional Products
PO Box 1
2600 MA Delft
The Netherlands
E-mail:
Tel: 31 152793592
Fax: 31 152793614

Local representative

N. Hendy
Technical Manager
DSM Food Specialties
9 Moorebank avenue
2170 Moorebank
Australia
E-mail: Neil.Hendy@DSM.com
Tel: 61 2 87789851
Fax: 61 2 98211442

2 Purpose of the application

It is the intention of DSM Food Specialties, The Netherlands to submit an application for the use of a new processing aid. This processing aid is an enzyme, more particular amylomaltase produced by a genetically modified microbial strain, *Bacillus amyloliquefaciens*.

The enzyme is not yet approved by FSANZ although other glucanotransferases from other microbial sources are.

Purpose of this application is to amend Standard 1.3.3. Processing Aids, table to clause 17([1]) as follows:

Amylomaltase EC 2.4.1.25	*Bacillus amyloliquefaciens,* containing the gene for Amylomaltase isolated from *Thermus thermophilus*

This application is made on behalf of the applicant DSM Food Specialties B.V.

3 Justification for the application

3.A. Need and/or advantages for the proposed change

Amylomaltase will be used to make Etenia™ out of potato starch by converting glucose units from amylose to amylopectine. Etenia™ (modified potato starch) has excellent thermoreversible gelling properties.

Typical applications in which Etenia™ can be used are yoghurts and yoghurt drinks, cheese analogues and low fat spreads. In low-fat spreads and cheese analogues, Etenia™ will be an alternative for other starch replacements of fat and casein, like gelatin.

By this change of the code, the producers of, for example, yoghurts, curds, mousses and ice creams may be able to replace fat and casein and other fat and casein substitutes in their products. As a consequence, the intake of fat by consumers could be reduced up to 2.5%.

3.B.1 The safety of the processing aid

The processing aid is safe as described in section II.D.

3.B.2 Nutritional issues related to the proposed change

Given the very small amount of enzyme to be added to the starch (3.8 units per gram of dry starch) and follow little amount (1.5-5.5%) of Etenia™ (modified potato starch) to the final food no relevant nutritional effects are foreseen.

3.C Technological need for the processing aid

Amylomaltase will be used to make Etenia™ out of starch by converting glucose units from amylose to amylopectine. Etenia™ has excellent thermoreversible gelling properties and it can thus replace fat and casein and other fat and casein substitutes in

[1]) In the Handbook called clause (o)

for example yoghurts, curds, mousses and ice creams. As a consequence, the intake of fat could reduce up to 2.5% as can be seen in Annex 3.1

Typical applications in which Etenia™ can be used are yoghurts and yoghurt drinks, cheese analogues and low fat spreads. In low-fat spreads and cheese analogues, Etenia™ will be an alternative for other starch replacements of fat and casein, like gelatin. Gelatin is animal derived and therefore not suitable in every type of product.

3.D. Potential impact on trade

The total impact of the use of this enzyme as processing aid will be relatively small, as the volume of enzyme preparation to be used is only 50-180 mg per kg or litre of final product.

It might however affect trade volumes of other native and modified starches and gelatin.

3.E. Consumer choice

Consumers will not notice the difference between products produced the current standard way or those produced with this enzyme. There will be no taste differences in the products after being produced with either method.

It is found that after gelling of Etenia™ about 10% Etenia™ is resistant starch. Resistant starch is generally known to be nutritional favorable due to a possible positive effect on risk reduction for colon cancer (Cummings 1996).

3.F. Interest of the industry

The enzyme is already used to produce potato modified starch Etenia™ in the Netherlands. There are also Australian producers have shown their interest in modified potato starch.

3.G. The costs and benefits for industry, consumers and government associated with use of the processing aid.

Etenia™ prepared with DSM's amylomaltase will be easier for processing and therefore gives benefits for the industry due to its excellent thermoreversible gel properties. That means that at ambient temperatures Etenia™ is a gel; and at higher temperatures Etenia™ behaves more like a liquid. Because of the formation of thermoreversible gels, Etenia™ can act as a fat mimic compound. Therefore Etenia™ can e.g. be used as a substitute of fat.

Another possible application of Etenia™ is the replacement of gelatin. From a safety point of view, the replacement of gelatin is good due to the fact that gelatin can possibly introduce BSE from animals to humans. Moreover gelatin can not be used in certain markets where kosher and halal products are required.

Generally spoken the replacement of fat by Etenia™ has a positive effect on the energy intake and could reduce the fat intake by consumer up to 2.5%.

Costs associated with the use of amylomaltase for consumers and industry will be the indirect and direct costs of the use of the enzyme and its implementation in the production process. For the government it will be the costs associated with the adaptation of its regulations.

4 Information to support the application

The safety of the product is discussed in section II.D; over the last 12 months significant sales took place in other countries by several globally operating companies. Companies are considering further implementation of Etenia™ around the world, including Australia and New Zealand.

5 Assessment procedure

According to DSM, this dossier should be assessed according the General Procedure, level 1 since it applies for the allowance of a similar class (group) enzyme as processing aid that is already permitted, but not yet in this micro-organism.

6 Confidential commercial information (CCI)

No confidential commercial information is incorporated in this dossier.

7 Exclusive capturable commercial benefit (ECCB)

The declaration of exclusive capturable commercial benefit is available as Annex I.7-1

8 International and other national standards

8.A. International Standards

The enzyme complies with the specifications for enzymes of both JECFA (JECFA, 2006) and the Food Chemicals Codex (FCC, 2010) as explained in Section II.B.5. There is no Codex standard for the enzyme, since there are no specific Codex standards for enzymes.

8.B. Other National Standards or Regulations

The enzyme activity of amylomaltase (transglucosidase or glucanotransferase) with I.U.B number 2.4.1.25 is listed in the Pariza and Johnson (2001) paper under “Enzymes used in food processing today”.

9 Statutory declaration

The statutory declaration is available as Annex I.9-1

10 Checklist

The checklist is available as Section III

II PROCESSING AIDS

A. Technical information on the processing aid

1. Information on the type of processing aid

Amylomaltase is an enzyme from microbiological origin, thus falling in category o.

2. Information on the identity of the processing aid

- Systematic name : (1→4)-α-D-glucan: (1→4)-α-D-glucan 4-α-D glycosyltransferase
- Accepted name : 4-α-glucanotransferase
- Common name : amylomaltase
- Other names : Disproportionating enzyme; dextrin glycosyltransferase; D-enzyme; debranching enzyme maltodextrin glycosyltransferase; dextrin transglycosylase,
- Marketing name : Meltamase™
- IUPAC/IUB Number : EC 2.4.1.25
- CAS number : 9032-09-1

Amylomaltase belongs to a family of hexosyltransferases, part of the glycosyltransferases.

The amino acid sequence of the enzyme is as follows:

MELPRAFGLLLHPTSLPGPYGVGVLGREARDFLRFLKEAGGRYWQVLPLGPTGYGDSPYQSFSAFAGNPY
LIDLRPLAERGYVRLEDPGFPQGRVDYGLLYAWKWPALKEAFRGFKEKASPEEREAFAAFRREREAWWLED
YALFMALKGAHGGLPWNRWPLPLRKREEKALREAKSALAEEVAFHAFTQWLFFRQWGALKAEAEALGIRII
GDMPIFVAEDSAEVWAHPEWFHLDEEGRPTVVAGVPPDYFSETGQRWGNPLYRWDVLEREGFSFWIRRL
EKALELFHLVRIDHFRGFEAYWEIPASCPTAVEGRWVKAPGEKLFQKIQEVFGEVPVLAEDLGVITPEVEALR
DRFGLPGMKVLQFAFDDGMENPFLPHNYPAHGRVVVYTGTHDNDTTLGWYRTATPHEKAFMARYLADWG
ITFREEEEVPWALMHLGMKSVARLAVYPVQDVLALGSEARMNYPGRPSGNWAWRLLPGELSPEHGARLRA
MAEATERL

- Host organism : *Bacillus amyloliquefaciens*
- Donor organism : *Thermus thermophilus*

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

Possible interactions with different foods

Starch is a polymer of glucose molecules. These molecules are bound on the α-1,4 binding site and/or α-1,6 linkage. Native starch is a combination of amylose and

amylopectin. Amylose does have mainly α-1,4 bonds which results in linear molecules of about 1000-6000 glucose units (Degree of Polymerization DP). Amylopectin does have additional α-1,6 bonds which results in branching of the molecules. The length of the branches is about DP 10-60.

Amylomaltase, the enzyme used for the production of Etenia™, breaks down α-1,4 linkages and in a second step catalyzes reactions with the formation of an other α-1,4 linkage. These results in two things: The amylose will be broken down and the length of the branches of the amylopectin will effectively increase. The side chain distribution of the amylopectin will also change, while these side chains will both be broken down and at the same time made longer by this enzyme.

This leads to the conclusion that Etenia™ is a special kind of potato starch which differs only in chain length distribution compared to potato starch and not in the primary structure and can thus be considered a normal constituent of the diet. There is therefore no basis to believe that other interactions of the enzyme have relevance to humans.

Enzymatic properties

The method to determine the activity of amylomaltase is based on the amount of glucose formed during incubation of maltotriose with amylomaltase under specific reaction circumstances. One amylomaltase unit (ATU) is defined as the amount of enzyme which produces 1 µmol of glucose per minute under the assay conditions of the test. This assay method is shown in Annex II.A.3-1.

The molecular weight (MW) of the enzyme, deduced from the amino acid sequence, is 57221 Da and on SDS-PAGE approximately 50 kDa.

Subsidiary enzymatic activities

Like any other living micro-organism, the production organism *Bacillus amyloliquefaciens* of this amylomaltase produces many other enzymes needed for the breakdown of nutrients and build up of cell material.

Although amylomaltase is being produced in excess, the enzyme preparation will also contain minor, non-standardized amounts of these other enzymes. These amounts do not have an effect (positive or negative) in the applications.

Particle data

The processing aid is not particulate.

4. Manufacturing process

DSM amylomaltase is produced by a controlled submerged fermentation of a selected, pure culture of *Bacillus amyloliquefaciens* (see Sections D and E). The production process includes the fermentation process, recovery (downstream processing) and

formulation of the product. An overview of the different steps involved is given in Annex II.A.4-1.

Fermentation process

The fermentation process consists of two steps: inoculum fermentations and main fermentation. The whole process is performed in accordance with Good Food Manufacturing Practice (see below).

Inoculum fermentations

During the first fermentation, a vial with a pure culture of a strain of the *Bacillus amyloliquefaciens* is suspended in purified water. For seeding, this suspension is aseptically transferred to a shake flask containing the following raw materials which are sterilized, safe and suitable for food production:

- Glucose
- Yeast extract powder
- Water
- Potassium phosphate
- Antifoam

Foaming is prevented by the addition of food-grade antifoam. This first phase inoculum is allowed to grow for circa 28 hours at 31°C.

Subsequently, the contents of the first phase inoculum are transferred to the seed fermentor.

This fermentor contains the following raw materials which are sterilized, safe and suitable for food production:

- Glucose
- Yeast extract
- Water
- Phosphoric acid
- Calcium chloride
- Other salts
- Antifoam

This inoculum fermentation is run for circa 25 hours at 34°C.

At the end of this fermentation the contents of the seed fermentor are used to start the main fermentation.

Main fermentation

Biosynthesis of amylomaltase occurs during the main fermentation. To produce the enzyme of interest, a submerged, aerobic fed batch fermentation process is employed, using a deep tank fermentor. This fermentor is equipped with devices for pH, temperature, oxygen and antifoam control, a top-mounted mechanical agitator and a bottom air sparger.

The contents of the seed fermentation are transferred to the main fermentor that already contains the following raw materials which are sterilized, safe and suitable for food production:

- Glucose
- Yeast extract
- Calcium chloride
- Salts and mineral nutrients
- Phosphoric acid and/or Sodium hydroxide for pH adjustment
- Antifoam

The fermentor is continuously fed by aseptically introducing sterilized medium (containing equivalent raw materials as the batch medium) according to a preset feeding programme.

During the fermentation of temperature, pH and other parameters are kept at set points.

After approximately 92 hours addition the fed batch stage is stopped and the fermentation will be finished.

Growth of the production organism and increase of enzyme production are regularly monitored in the main fermentation by analysis of aseptically collected samples.

Downstream processing

The downstream processing consists of the following steps:

- Killing the production strain
- Removal of the cell material
- Active carbon treatment
- Ultrafiltration
- Stabilization/Storage

Killing off

After the fermentation is stopped, the production organism is killed by addition of sodium di acetate.

The amylomaltase is released from the cells by applying a homogeniser to mechanically disrupt the cells.

Removal of the cell material

The pH of the broth is increased to pH 9 with NaOH and calcium chloride; in addition a filter aid and a flocculant are added. Then, the fermentation broth is filtrated using a membrane filter press.

Active carbon treatment

The material is treated with active carbon in order to reduce the antifoam load. Consequently, the broth is polish filtrated and sterilised with a germ filtration.

Ultrafiltration

The final step in the recovery is a combination of ultrafiltration and diafiltration to concentrate the product and consequently remove the di acetate. During this ultrafiltration, molecules with a molecular weight smaller than 5 –10 kD (depending on the shape of the molecules) are removed from the broth. The high molecule weight

fraction of the proteins is concentrated, whilst the concentration of the low molecule weight fraction remains more or less the same.

Stabilisation/Storage

The UF concentrate is stabilised with glycerol again polish and germ filtrated and subsequently stored at 4 – 6°C until further handling.

Stabilisation and Formulation

In order to obtain an end product, the UF concentrate is formulated with glycerol after which more glycerol is used to bring the product to the desired amylomaltase activity of 1000 ATU/ml.

Total Organic Solids (TOS)

The Total Organic Solids of the enzyme preparation were calculated from non-stabilized and un-standardized samples of 3 different fermentation batches, sampled before the required second UF concentration (see Section II.B.4).

Calculation of the TOS					
Batch number	Water (%)	Ash (%)	TOS (%)	Activity (ATU / g)	ATU / g TOS
1	87.7	2.17	10.1	4200	41600
2	93.24	1.26	5.50	2130	38727
3	79.9	3.52	16.6	5930	35700
MEAN					38676

HYGIENE

Good Food Manufacturing Practice

For optimal enzyme production, it is very important that hygienic conditions during the whole fermentation process are strictly controlled. Microbial contamination would immediately result in less growth of the production organism and consequently in a low yield of the wanted enzyme(s).

In addition to the microbial hygiene, it is also important that the raw materials and processing aids used during fermentation are of sound quality and do not contain contaminants such as pesticides or a high amount of heavy metals, which might affect the optimal growth of the production organism and thus enzyme yield.

Of course, the quality of the stock culture and the strict control of parameters as pH, temperature and aeration during fermentation are also of the utmost importance for optimal enzyme production and yield.

Thus, the commercial self-interest of any enzyme producer demands a strictly controlled fermentation process.

Enzyme fermentation experience in the DSM factory at Capua, Italy, has resulted in a solidly established Good Food Manufacturing Practice within the framework of a certified ISO system.

Technical measures:

The batches of **primary seed material**, also called Working Cell Bank (WCB), are always prepared from the so-called Master Cell Bank (MCB) in Laminar Air-flow (downflow) safety cabinets to ensure the absence of contamination. The batches are divided into a large number of vials for use in production over a long period of years without any changes in strain- and production properties. In theory, a batch is large enough to last for about 10 years, depending on the strain viability and the fermentation frequency and thus the market demand.

The above procedures for preparation, preservation and storage are chosen to avoid degeneration and to secure genetic stability. All vials are clearly labelled and in revival of the culture, strict aseptic techniques are applied.

The **raw materials** used to make up the nutrient medium for the fermentation are added to mixing tanks and sterilised. The heat-treated nutrient solutions are then cooled for optimum cell growth and subsequently transferred to the fermentor.

The **fermentor** is a closed system. Air introduced into the fermentor is sterilised with a filter. Proper temperature conditions are maintained with cooling coils inside the fermentor.

Prior to inoculation, the fermentor is cleaned with solutions of food grade detergents, rinsed with water and then sterilised (empty) with steam.

All materials are pumped into the fermentor under overpressure via fixed connections which are equipped with self-closing valves. In this way, the sterilised nutrient medium

from the mixing tank and the complete biomass broth from the inoculum fermentation are transferred aseptically to the main fermentor.

Microbial contamination during **fermentation** is prevented by the use of a large inoculum, carefully chosen optimum growth conditions for the production organism, overpressure in the fermentation vessel, and the use of sterile air.

The germ filtration during **downstream processing** additionally ensures that the end product is free of microbial contamination.

Control measures:

A new WCB is prepared from the MCB as soon as the previous batch becomes depleted or the concentration of viable cells decreases.

After preparation of a new WCB, samples are checked for identity, viability and microbial purity, using different temperatures (25, 30 and 37°C) and media, by enrichment and viewing morphology (colony shape and microscopy). If all these parameters are correct, the strain is tested for production capacity, first on laboratory scale and later on large scale production level. Only if the productivity and the product quality meet the required standards, the new WCB is accepted for further production runs.

The viability of the WCB is checked at least once a year.

The **raw materials** used in the fermentation process are checked to be of suitable purity and free of harmful substances. The ingredients used are tightly controlled to minimize the risk of contaminants that would inhibit growth of the production organism or enzyme production.

The Quality Control (QC) Department provides assurance that these materials comply with appropriate specifications.

During the **seed fermentation** manual samples are taken aseptically from a sampling port on the fermentor for analysis in the laboratory. Samples are checked for pH and microbiological quality.

During the **main fermentation** the correct temperature, pH and dissolved oxygen content are monitored and automatically adjusted throughout the process to ensure optimal enzyme production and a consistent process and thus product.

During the main fermentation manual samples are taken aseptically from a sampling port on the fermentor for analysis in the laboratory. These samples are analysed for such parameters as microbial purity, pH, viscosity and enzyme activity. If microbial controls show that contamination has occurred, the fermentation will be discontinued.

Also during **downstream processing,** most particularly at the end of the UF concentration, samples are taken and checked for activity, dry matter, pH, specific gravity and the level of microbial contamination.

After **stabilisation**, the semi-finished product is analysed for colour, amylomaltase activity, dry matter content, and particle size and checked for microbiological contamination. Only if the product meets the in-process specifications, it will be accepted as a basis to formulate the final commercial product.

5. Specification for identity and purity

The final enzyme preparations are analysed for the following parameters:

Parameter	**Norm**
Amylomaltase activity	1000 +/- 5% ATU/g
Appearance	Light yellow to brown liquid
pH	6.5 – 7.5

In addition, according to the general specifications for enzyme preparations used in food processing as established by the Joint Expert Committee of Food Additives of the FAO/WHO (JECFA, 2006), The Food Chemical Codex 7th edition and according to the French legislation (Arrêté, 2006), the amylomaltase preparations from *Bacillus amyloliquefaciens* fulfil the following demands:

Parameter	**Norm**
Lead	≤ 5 mg/kg
Cadmium	≤ 0.5 mg/kg
Mercury	≤ 0.5 mg/kg
Arsenic	≤ 3 mg/kg
Standard plate count	≤ 5x10^4/g
Coliforms	≤ 30/g
Salmonella	0/25 g
Escherichia coli	0/25 g
Anaerobe sulphite reducing	< 30/g
Staphylococcus aureus	0/g
Antimicrobial activity	Absent by test
Mycotoxins	Absent by test

Total Microbial Count

As is explained above and proven below, the amylomaltase preparation complies with international purity standards and addition to foodstuff (in an amount of maximally 1% on basis of w/w) will therefore not cause an increase in the total microbial count.

Heavy metals

As can be seen in the 3 Certificates of Analysis given in Annex II.A.5-1, the amylomaltase preparations comply with the specifications for heavy metals as recommended by JECFA.

Microbiological contaminants

As can be seen in the 3 Certificates of Analysis given in Annex II.A.5-1, the amylomaltase preparations comply with the specifications for microbial contaminants as recommended by JECFA.

Test for absence of the production strain

For proprietary reasons, it is very important for each enzyme producer that the final commercial product does not contain viable production organisms. In the case of amylomaltase production, the cells are killed off at the end of the fermentation (see Section II.A.4), which ensures that the final product is free from the production organism *Bacillus amyloliquefaciens.*

Test for absence of antibiotic activity

As can be seen in the 3 Certificates of Analysis given in Annex II.A.5-1, the amylomaltase preparations do not contain antibiotic activity.

Test for absence of toxins

Bacillus species are not known to produce mycotoxins. According to JECFA, only enzymes from fungal origin have to be tested for mycotoxins. Besides this, *Bacillus subtilis* (now named *Bacillus amyloliquefaciens*) has been identified as an organism which is negative for toxin production, see Annex II.A.5-2.

Further analysis on the final enzyme preparations for (myco)toxins has therefore not been performed.

Known allergens

The formulation the amylomaltase is sold in does not contain allergens.

Presence of residues in the final product

During the production process of Etenia™, the amylomaltase is added to the suspension of potato starch. After desired viscosity of starch solution is achieved the solution is processed by a jet-cooker at 120 ^{o}C to inactivate the enzyme. Finally starch solution is spray-dried. Therefore no active enzyme will be present in the final food.

C. Information related to the safety of an enzyme processing aid

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

The amylomaltase currently is used only in the Netherlands to modify the potato starch and this enzyme modified potato starch is used worldwide. Currently, the use of amylomaltase is considered as processing aid in EU and no formal approval is required provided enzymes are safe.

The modified potato starch with help of alylomaltase can be considered GRAS in USA based on GRAS self affirmation prepared by DSM that was confirmed by an independent expert panel.

2. Information on the toxicity of the enzyme processing aid

The FAO/WHO Joint Expert Committee on Food Additives (JECFA) has evaluated the safety of mixed microbial carbohydrase and protease from *Bacillus subtilis* (now named *Bacillus amyloliquefaciens)* already in 1971. A toxicological monograph has been published a year later.

Based on adequate toxicological data and on the fact that *Bacillus subtilis* occurs ubiquitously and is a common contaminant of food, JECFA established an unlimited Acceptable Daily Intake (ADI) for α-amylase and mixed microbial carbohydrase and protease from *Bacillus subtilis*.

In the USA a carbohydrase preparation from *Bacillus subtilis*, characterized by the presence of the enzymes α-amylase and ß-glucanase was affirmed as GRAS (generally recognized as safe), with no limits other that those in good manufacturing practice (cGMP). Carbohydrase enzyme preparations and protease enzyme preparations derived from *Bacillus amyloliquefaciens* were also affirmed as GRAS, respectively 21 CFR §184.1148 and 21 CFR §184.1150.

Apart from the positive evaluation of JECFA and GRAS-notifications in the USA, most countries that regulate the use of enzymes, such as France, Denmark, Australia and Canada, have accepted the use of enzymes from *Bacillus amyloliquefaciens* in food applications.

Based on the above mentioned information, it is concluded that enzymes from *Bacillus amyloliquefaciens* are safe for use in food.

Consumer safety of enzyme preparations is determined usually by three variables: the producing organism, the raw materials used in the production process and the production process itself. In certain cases the enzyme might be of concern as well. The safety of the production process is embedded in current Good Manufacturing Practice (cGMP) and Hazard Analysis of Critical Control Points (HACCP). The recipe of the production process of any new production strain is kept constant with respect to the raw materials composition. The enzyme activity of amylomaltase (transglucosidase or

glucanotransferase) with I.U.B number 2.4.1.25 is listed in the Pariza and Johnson (2001) paper under “Enzymes used in food processing today”. The amylomaltase produced by Bacillius subtilis containg gene from Thermus aquaticus was evaluated for its safety by S. Tafazoli, A. W. Wong and etc. (2001) and it was concluded that this enzyme is safe for food production. Consequently, the background of the production organism determines the safety of the new enzyme product.

As is outlined in Section D the *Bacillus amyloliquefaciens* lineage used for the production of amylomaltase has been in use by DSM in large-scale fermentations since the sixties. In particular strains from this lineage are in use for the production of the enzymes α-amylase, neutral protease and ß-glucanase. *Bacillus amyloliquefaciens* has been generally accepted as a nonpathogenic organism and the non-toxicogenicity has been confirmed by a large amount of toxicological tests on enzymes derived from the species. The parent strains EBA-127 and EBA-1 of the current amylomaltase producing strain MAS-3 were declared suitable host strains for the construction of genetically modified organisms belonging to Group I safe micro-organisms by the Dutch competent authorities (see Annex II.D.2-2 and II.D.2-3). The recombinant *Bacillus amyloliquefaciens* strain MAS-3, which differs from its parent EBA-127 only in the presence of some additional amylomaltase genes through the presence of the pGBB05MAS1 vector, has been classified both by the Dutch and the French competent authorities as a genetically modified micro-organism that can be used safely for large-scale production (see Annex II.D.2-4 and II.D.2-5 respectively). Based on the genetic modifications performed, there are no reasons to assume that the recombinant production strain should be less safe than the host or its original classical parent. The production strain only overproduces the amylomaltase enzyme whereas the ancestral parental strain does not.

Apart from its history of safe use, *Bacillus amyloliquefaciens* has a Qualified Presumption of Safety (QPS) status.

In the opinion of the EFSA Scientific Committee regarding the QPS approach for assessment of selected microorganisms, which was adopted by EFSA in November 2007, *Bacillus amyloliquefaciens* along with other Bacillus species, was recommended for QPS status provided absence of emetic food poisoning toxins with surfactant activity and absence of entertoxic activity can be demonstrated.

In 2003, Amfep initiated a study on specific toxin-analyses with many industrial *Bacillus* strains, including 5 *Bacillus amyloliquefaciens* strains, among which the DSM strain EBA-1. The analyses showed that none of the industrial strains has the potential to produce toxins under the conditions. In 2004, EFSA confirmed the position of the Amfep regarding *Bacillus amyloliquefaciens Bacillus amyloliquefaciens* therefore fulfills the requirements for QPS.

Based on the above, it is concluded that *Bacillus amyloliquefaciens* is considered a safe microorganism for the production of amylomaltase.

According to EFSA guidance document on the submission of dossiers for food enzymes (2009), toxicological data provided can be reduced or even completely waived for food enzymes produced by micro-organisms with a QPS status, if it can be demonstrated that there are no concerns related to any residues, degradation products or substances originating from the total production process.

As *Bacillus amyloliquefaciens* has a QPS status, there is no requirement to perform toxicological studies.

Nevertheless, in order to confirm the toxicological safety of the use of amylomaltase from *Bacillus amyloliquefaciens* in food, the following studies were performed:

- subacute (14-days) oral toxicity study

- subchronic (90-days) oral toxicity study

- Ames test, *in vitro*

- chromosomal aberration test, *in vitro*

All studies were performed according to international accepted guidelines (OECD/ EU) and are in compliance with the principles of Good Laboratory Practice (FDA/OECD).

The test material (batch MEG.GRZ.0905, referred to as 'tox-batch') was an unformulated UF concentrate produced according the procedure used for the commercial preparation, see Section A.4. It contained an activity of 2130 ATU/g and a TOS content of 5.50%. The Certificate of Analysis is included in the oral toxicology report.

Summarizing the results obtained from the toxicity studies performed, the following conclusions can be drawn:

- The tox-batch did not show any mutagenic or clastogenic activity under the given test conditions.

- The results of the sub-chronic (90-day) oral toxicity study lead to a No Observed Adverse Effect Level (NOAEL) of 18182 mg/kg bw/day, the highest dose level tested. This amount of enzyme preparation is equivalent to an intake of 38728 ATU/kg bw/day or 1000 mg TOS/kg bw/day.

Based on the NOAEL and the maximum amounts of enzyme present in the final food (see Section 6.1), the Margin of Safety (MoS) for human consumption can be calculated using two different ways of estimating the daily consumption of the enzyme:

Theoretical Maximum Daily Intake (TMDI)

Using the Budget method (Douglass et al, 1997) and the data given in Section II F.2 the Theoretical Maximum Daily Intake (TMDI) of amylomaltase in food will be: TMDI =

ATU/kg food/80[2] = 209/80 = 2.61 ATU/kg bw/day. This calculation is based on the highest dose level.

Estimated Daily Intake (EDI)

The EDI was calculated based on the estimated consumption of Etenia™ used as fat replacer (Jansen et al., 2006). The 95th percentile for intake of Etenia™ is estimated to be 8.6-11.8 g/day. The highest intake is found in the children category 1-3 years; it results in an intake of 8.9 g/day for an 8 kg child, leading to an intake of 1.1 g/kg bw/day.

Based on the data given in Section II F.2, the following calculation can be made:

Residual amount of (inactivated) enzyme in final food (ATU/kg)	Estimated daily intake (g food/kg bw/day)	Estimated daily intake of (inactivated) enzyme (ATU/kg bw)
57 - 209	1.1	0.063 – 0.23

The Margin of Safety (MoS) can be calculated by dividing the NOAEL by either the TMDI or the EDI. With an overall NOAEL of 38728 ATU/kg bw/day the following MoS can be calculated:

MoS based on TMDI	Minimal MoS based on EDI
38728 / 2.61 = 14838	38728 / 0.23 = 168383

Regarding the height of the MoS, it was concluded that further testing of the safety of the product is not meaningful.

[2] This calculation is based on the assumption that daily consumption of amylomaltase containing foods will usually not exceed half of the assumed maximum total solid food intake (i.e. 12.5 g/kg bw/day (1/80 kg food/kg bw/day)). According to the Budget method, the maximum daily food intake is 25 g/kg bw/day (1/40 kg food/kg bw/day).

14-days oral toxicity

A dose-range finding (14 day) oral toxicity study with the tox-batch was conducted at Advinus, India, in order to assess the systemic toxicity potential of the test item when administered orally by gavage to rats and also to determine the doses to be used for the subsequent 90-day oral toxicity study.

The study was conducted in accordance with the OECD Guideline for the Testing of Chemicals 407. Repeated Dose 28-day Oral Toxicity Study in Rodents, adopted 3rd October 2008.

The study comprised of four groups of 6 male and 6 female Wistar rats, one control group and three test groups (low, mid, and high dose). For a period of 14 consecutive days, the test material was administered daily by gavage at concentrations of 1818 (low), 5454 (mid) and 18182 (high) mg per kg body weight per day. The animals in the control group received vehicle (water) only. The low, mid, and high dose correspond with respectively 100, 300 and 1000 mg TOS (Total Organic Solids)/kg body weight/day.

The following parameters were evaluated in all animals:

- Clinical observations
- Body weight
- Food consumption
- Haematology
- Clinical chemistry
- Organ weights (thymus, epididymis, brain, heart, adrenals, gonads, spleen, liver, kidneys, ovaries, uterus and testes)
- Macroscopy at necropsy
- Microscopy of stomach (control and high dose) and all gross lesions

Results

- 100 mg TOS/kg/day: no treatment-related findings noted
- 300 mg TOS/kg/day: no treatment-related findings noted
- 1000 mg TOS/kg/day: no treatment-related findings noted

Conclusion

The administration of the tox-batch at levels up to 18182 mg/kg bw/day did not result in treatment related effects in the rats (see Annex II.C.2-1).

90-days oral toxicity

A sub-chronic (90 day) oral toxicity study with the tox-batch was conducted at Advinus, India, in order to assess the systemic toxicity potential of the test item when administered orally by gavage to rats.

The study was conducted in accordance with the following guidelines:

• OECD Guideline for the Testing of Chemicals 408. Repeated Dose 90-day Oral Toxicity Study in Rodents, adopted 21st September 1998.

• B.26. Subchronic oral toxicity test. Repeated dose 90-day oral toxicity study in rodents. Annex 5D to Commission Directive 2001/59/EC, Official Journal of the European Communities L225, 21.8.2001.

The study comprised of four groups of 10 male and 10 female Wistar rats, one control group and three test groups (low, mid, and high dose). For a period of 90 consecutive days, the test material was administered daily by gavage at concentrations of 1818 (low), 5454 (mid) and 18182 (high) mg per kg body weight per day. The animals in the control group received vehicle (water) only. The low, mid, and high dose correspond with respectively 100, 300 and 1000 mg TOS (Total Organic Solids)/kg body weight/day.

The following parameters were evaluated in all animals:

- General clinical observations
- Physical/detailed clinical observations
- Ophthalmological examination
- Neurological examination
- Body weight
- Food intake
- Haematology
- Clinical chemistry
- Urinanalysis
- Organ weights (adrenals, brain, epididymides, heart, liver, ovaries, pituitary gland, prostate, spleen, testes, thymus, thyroid and uterus)
- Macroscopy at necropsy
- Histopathology of organs (control and high dose) and microscopy of all lesions

Results

- 100 mg TOS/kg/day: no treatment-related findings noted
- 300 mg TOS/kg/day: no treatment-related findings noted
- 1000 mg TOS/kg/day: no treatment-related findings noted

Conclusion

The administration of the tox-batch at levels up to 18182 mg/kg bw/day did not result in treatment related effects in the rats. 18182 mg/kg bw/day, which is equivalent to 1000 mg TOS/kg bw/day or 38728 ATU/kg bw/day, is considered as the NOAEL (see Annex II.C.2-2).

Mutagenicity tests

AMES test (see Annex II.C.2-3)

A bacterial reverse mutation test was performed with the tox-batch at TNO, the Netherlands, in order to assess its mutagenic activity in four selected strains of *Salmonella typhimurium*, TA 1535, TA 1537, TA 98 and TA 100, as well as in the *Escherichia coli* mutant WP2 *uvrA*. All were tested in both the absence and presence of a metabolic activation system (S9-mix).

The study was conducted in accordance with OECD guideline no. 471, Genetic toxicology: Bacterial Reverse Mutation Test, adopted 21st July 1997.

One bacterial reverse mutation test was performed in the absence and presence of S9-mix with five concentrations of the tox-batch, ranging from 62 to 5000 μg/plate. Negative controls (water) and positive controls were run simultaneously with the tox-batch.

The negative control values were within the acceptable range and strain-specific positive controls gave the expected increase in the mean number of revertant colonies.

The tox-batch was slightly toxic to strain TA1537 in the absence of S9-mix at 1667 μg/plate and 5000 μg/plate and in the presence of S9-mix at 5000 μg/plate, as evidenced by a small clearing of the background lawn of bacterial growth compared to the negative controls. As only slight toxicity was observed, no decrease in the mean number of revertants was observed and at least 3 concentrations of the tox-batch were non-toxic, the test was regarded as valid.

In all strains and in both the absence and the presence of S9-mix, the test substance did not cause a significant dose-related increase in the mean number of revertant colonies compared with the negative control.

It is concluded that the tox-batch was not mutagenic under the conditions employed in this study.

Chromosomal aberration test (see Annex II.C.2-4)

A chromosomal aberration test *in vitro* was performed with the tox-batch at TNO, the Netherlands, in order to assess its ability to induce structural chromosomal aberrations in cultured human lymphocytes in the absence and presence of a metabolic activation system (S9-mix).

The study was conducted in accordance with the following guideline

OECD guideline 473, Genetic toxicology: *In vitro* Mammalian Chromosome Aberration Test, adopted 21st July 1997.

Two separate tests were conducted. Dose levels of the tox-batch ranging from 10 to 5000 μg/ml were tested. Negative controls and adequate positive controls were run simultaneously.

In the first test cells were treated/ harvested for 4/24 hours (pulse treatment) in both the absence and presence of S9-mix. Three dose levels of the tox-batch were tested: 1250, 2500 and 5000 μg/ml.

In the second test, the treatment/harvesting times were 4/24 hours (pulse treatment) and 24/24 hours (continuous treatment) in the presence and absence of S9-mix. Three dose levels of the tox-batch were tested: 1000, 3000 and 5000 μg/ml.

The incidence of structural chromosomal aberrations found in the negative (vehicle) controls, was within the historical range. The positive control substances mitomycin C (in the absence of the S9-mix) and cyclophosphamide (in the presence of the S9-mix) induced the expected increases in the incidence of structural chromosomal aberrations.

The tox-batch did not induce a statistically significant or biologically relevant increase in the number of cells with chromosome aberrations in the absence and presence of S9-mix in either of the two tests.

It is therefore concluded that the tox-batch was not clastogenic to cultured human lymphocytes under the conditions employed in this study.

D Additional information related to the safety of an enzyme processing aid derived from a micro-organism

1. Information on the source micro-organism

Identification and Taxonomy

The classical parent EBA-1 and the recombinant strain, the industrial production strain MAS-3, have been taxonomically identified by taxomical experts of respectively the TU Delft (The Netherlands) and DSMZ Braunschweig (Germany). These are independent, internationally recognised laboratories.

As can been seen in Annex II.D.1-1, the classical strain EBA-1 (DS 19573) as well as its parent BZ-53 (DS 3225) were determined as *Bacillus amyloliquefaciens.* Also the recombinant strain, the protease production strain MAS-3 was determined as *Bacillus amyloliquefaciens* (see Annex II.D.1-2).

The species *Bacillus amyloliquefaciens* is closely related to *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus.* Since *Bacillus amyloliquefaciens* and *Bacillus subtilis* are difficult to separate on the basis of the classical phenotypic tests alone, *Bacillus amyloliquefaciens* generally was classified as *Bacillus subtilis*. Presently however, these two species can be separated using probabilistic identification methods based on API-tests, pyrolysis gas-liquid mass spectrometry, DNA-homology and on the molecular % G + C of the DNA. Consequently, *Bacillus amyloliquefaciens* has now been given a separate species status and its name has been included on the approved lists of bacterial names (F.G. Priest et al., 1987).

Bacillus amyloliquefaciens is classified as follows:

Kingdom	:	Procaryotae
Division	:	Bacteria
Order	:	Bacillales (endospore-forming rods and cocci)
Family	:	Bacillaceae
Genus	:	Bacillus
Species	:	Bacillus amyloliquefaciens

Origin of the strain

The parental organism of the European *Bacillus amyloliquefaciens* (EBA) lineage was originally isolated in 1958 from nature in a screening programme and introduced into production in the plant of Gist-brocades (now DSM) in Seclin (France) soon thereafter. In 1965 the strain was re-isolated from a production batch.

Through mutation, using common mutagens such as UV radiation and nitrosoguanidine, followed by selection for enhanced enzyme production, the strain BZ-53 (DS 3225) was isolated in 1982. From BZ-53, using classical mutation (UV) and selection techniques, an asporogenous strain EBA-1 (DS 3229) (frequency of revertants of 1 x 10^{-8}) was isolated possessing an enhanced production of the enzymes alpha-amylase, neutral protease, and β-glucanase.

The *Bacillus amyloliquefaciens* EBA-lineage has been used by Gist-brocades (now DSM) in large scale fermentation tanks for the production of amylase, β-glucanase and protease, enzymes which are utilised world-wide in the beverage and baking industry from the sixties till to-day.

By using recombinant DNA technology the gene coding for alpha-amylase was deleted from strain EBA-1, resulting in the amylase deficient strain EBA-112 (DS 19294). Subsequently it was decided to knock out the genes coding for the major proteases, the neutral metalloprotease *NprE* and the alkaline protease *aprA*. The first gene was inactivated using classical mutagenesis, whereas the alkaline protease was eliminated by using rDNA technology. Again by using recombinant DNA technology, several amylomaltase encoding genes were inserted into EBA-127, resulting into the final production strain MAS-3.

Annex II.D.1-3 shows the different steps that were involved.

2. Information on the pathogenicity and toxicity of the source micro-organism

Most information on *Bacillus amyloliquefaciens* is basically information on *Bacillus subtilis* as during most studies the distinction between these species is not made. Bacteria related to *Bacillus subtilis* are commonly found in soil and from the soil they are transferred to various environments such as plants, plant materials, foods, animals and marine and freshwater environments. *Bacillus subtilis*-like organisms grow aerobically at intermediate temperatures and pH. They secrete substantial amounts of hydrolytic enzymes such as amylases and proteases and take part in the breakdown of organic materials.

For several decades, *Bacillus amyloliquefaciens* has been safely used in the commercial production of various food enzymes, such as amylase, protease, beta-glucanase and hemicellulase.

This long experience of industrial use has resulted in a good knowledge of the characteristics of *Bacillus amyloliquefaciens* and understanding of metabolic reactions.

The long industrial use and wide distribution of *Bacillus amyloliquefaciens* in nature has never led to any pathogenic symptoms. Moreover, no case demonstrating invasive properties of the species has been found in the literature (A. S. de Boer and B. Diderichsen, 1991). *Bacillus amyloliquefaciens* is therefore generally accepted as a **nonpathogenic** organism.

Even though products from *Bacillus amyloliquefaciens* have been used in food for many decades, there is no evidence that this species produces toxins. The **non-toxicogenicity** has been confirmed by a large amount of toxicological tests on enzymes

derived from the species, including recombinant strains (see JECFA evaluations in Annex II.D.2-1).

Apart from the positive evaluation of JECFA, most countries which regulate the use of enzymes, such as the USA, France, Denmark, Australia and Canada, have accepted the use of enzymes from *Bacillus amyloliquefaciens* in food applications.

The recombinant *Bacillus amyloliquefaciens* strain EBA-127, which differs from its parent EBA-1 by the absence of the active genes encoding for α-amylase, neutral metalloprotease and alkaline protease, and EBA-1 itself, was declared a suitable host strains for the construction of genetically modified organisms belonging to Group I safe micro-organisms (see Annex II.D.2-2) and a GMO obtained by self-cloning (see see Annex II.D.2-3).

The recombinant *Bacillus amyloliquefaciens* strain MAS-3, which differs from its parent EBA-127 only in the presence of some additional amylomaltase genes through the presence of the pGBB05MAS1 vector, have been classified both by the Dutch and the French competent authorities as a genetically modified micro-organism that can be used safely for large scale production (see Annex II.D.2-4. and II.D.2-5 respectively). Consequently it was introduced as the production organism for amylomaltase in the DSM factory in Capua, Italy.

Based on the genetic modification performed (see Sections E), there are no reasons to assume that the recombinant production strain should be less safe than the host or its original classical parent. The production strains only overprduces the amylomaltase enzyme whereas the ancestral parental strain does not.

The recombinant production organism is asporogenic and at the end of the fermentation, the cells are effectively killed off.

3. Information on the genetic stability of the source organism

The growth, morphology and production characteristics of the production strain can easily be maintained under normal laboratory and production conditions. At a low frequency abnormal colonies can be isolated as is routinely seen after plating highly specialized, industrial strains.

The recombinant strain stable overproduces the amylomaltase for over 60 generations as observed by protease productivity measurements as well as by analysis of the strain for neomycin resistance, which is indicative of the presence of the plasmid. No experimental data are available regarding the plasmid copy number, but in analogy with other pUB110 derived plasmids this is estimated as 50-100 copies per cell. At the end of the fermentation more than 90% of all analyzed colonies are neomycin resistant. The neomycin resistance naturally occurs on the plasmid pUB110, which is an endogenous *Bacillus* plasmid.

Plasmid pUB110 is not self-transmissable, but may be mobilized in the presence of self-transmissable plasmids. This requires an intact *mob*-gene, encoded by pUB110. However, the *mob*-gene in vector pGBB05MAS1 has been inactivated by insertion of the *masQ*-gene at this position. This results in a non-mobilizable genotype of the strain and therefore excludes any possibilities of transfer of the vector to other bacteria. The physical containment of the genetically modified strain in the enzyme production process a priori will minimize the chances of gene transfer.

E Additional information related to the safety of a processing aid derived from a genetically-modified micro-organism

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

Below, it is described how the host strain EBA-127 was obtained by a combination of classical mutation and genetic modification of the *Bacillus amyloliquefaciens* strain EBA-1.

The purpose of the genetic modification was to create an α-amylase deficient strain.

First, the gene encoding α-amylase, obtained from the parent *Bacillus amyloliquefaciens* strain BZ-53, was cloned as a 2.2 kB BamHI-BgII DNA fragment into the plasmid pUB110. pUB110 is an endogenous *Bacillus* plasmid and is a certified plasmid by NIH (USA). The complete nucleotide sequence as well as the functions of the different genes, including a gene for neomycin resistance (neo^r), encoded by the plasmid are known. The biology of pUB110 and related Bacillus plasmids, such as the also certified plasmid pE194, is very well studied (see Annex II.E.1-1).

From the resulting vector, called pUBBZ, a 4.1 kB XbaI-SnaBI fragment containing the entire coding sequence of the α-amylase gene was isolated. In addition, a 3.9 kB XbaI-SnaBI fragment of the plasmid pE194, containing the gene for neomycin resistance and a temperature senstive origin of replication (ts-ori), was isolated. These two fragments were fused to a 8 kB vector called pEAmy. By treating the pEAmy vector with the restriction enzymes EcoRV and HindIII, a 735 base-pair deletion was made in the α-amylase gene, yielding the 7.2 kB vector pEΔAmy with a non-functional α-amylase gene.

The vector pEΔAmy was used to transform the *Bacillus amyloliquefaciens* strain EBA-1. Transformants were selected on basis of their neomycin resistance under temperature conditions which were non-permissive for plasmid replication due to the ts-ori function. In this way, only those transformants are selected which contain the pEΔAmy vector integrated into the chromosomal α-amylase locus by homologous recombination.

Subsequently, the transformants were grown in the absence of neomycin in order to stimulate the “out-recombination” and curing of the plasmid sequences containing the neo^r and ts-ori elements. As a result a neomycin sensitive clone with a non-functional α-amylase gene (deletion of 735 base pairs) could be selected. This strain was designated EBA-112.

Next the strain EBA-112 was subjected to UV mutagenesis, following standard procedures. The surviving cells were plated onto 2xTY agar plates supplemented with 1% skim milk. The resulting colonies were visually inspected for a reduced halo formed as a result of extracellular protease production. It is known from previous studies (Vehmaanperä *et al*., 1991) that mutation or deletion of the *nprE* gene in *B. amyloliquefaciens* results in significantly reduced degradation of skim milk. One such colonies, showed a clear reduction in halo size and was subsequently analyzed biochemically for the absence of the NprE protein by SDS-PAGE and LC/MS/MS. From these data it was established that this strain is indeed completely NprE deficient. Growth

of this strain, designated EBA126, both in liquid cultures and on solid media, is identical to that of the ancestral strain EBA112.

Finally, the alkaline serine protease encoding gene, *aprA*, was deleted from EBA126 using a GMO approach. First, the flanking sequences of the *aprA* gene were amplified and fused by PCR, and cloned into plasmid pGBD1. This plasmid is a derivative of the well-documented plasmid pE194 (see Annex II.E.1-1), and contains (i) an origin of replication and selectable marker (Amp^R) for cloning in *E. coli*, derived from the commercial pCR2.1-TOPO vector (Invitrogen), and (ii) an additional marker for selection in bacilli derived from pUB110, which confers resistance to both neomycin and kanamycin (Neo/Kan^R). However, since the *E. coli* elements are inserted into the Neo/Kan^R gene, the latter is dysfunctional. The resulting plasmid was subsequently digested with the restriction enzyme *Bgl*II and the fragments containing the pE194 and *aprA* flanking sequences were re-ligated. The resulting ligation mix was used to transform competent cells of *B. subtilis* 1A40, selecting for resistance to kanamycine. The resulting transformants all contained the correct plasmid, pGBBdapr1, in which the *E. coli* sequences are deleted and the Neo/Kan^R marker is functionally restored. The construction of this plasmid is depicted in see Annex II.E.1-2.
Next, the plasmid was used to transform *B. amyloliquefaciens* EBA126 by electroporation. Since the pE194 origin of replication in thermosensitive, the plasmid was subsequently integrated into the chromosome of EBA126 by elevating the growth temperature to 48ºC, maintaining selective pressure on the Neo/Kan^R marker. Homologous recombination directed by the *aprA* flanking regions allowed for integration into the *aprA* locus, which was verified by means of PCR and Southern hybridizations using an *aprA* specific probe. One of the positive clones was subsequently used for excision of the integrated plasmid by growth at a temperature permissive for plasmid replication, in the absence of selective pressure. This resulted in a kanamycin sensitive progeny containing either the wild-type *aprA* gene, or a deletion of the gene (see also Annex II.E.1-3). The *aprA* deletion, as well as absence of the Neo/Kan^R marker was confirmed by Southern blot and or PCR analyses (see Annex II.E.1-4).
The final strain *B. amyloliquefaciens* EBA127 thus has the genotype *spo*$^-$, Δ*amy*, *nprE*$^-$, Δ*apr*A. The absence of the two major extracellular proteases was visualized by plating onto skim-milk containing agar plates (see Annex II.E.1-5). Growth of this strain, both in liquid cultures and on solid media, is essentially identical to that of the ancestral strains EBA112 and EBA126.

Below, a summary of the steps involved in the further genetic modification of the host EBA-127 to obtain the amylomaltase production strain is given.

Donor DNA

The gene encoding amylomaltase was made synthetically based upon the *malQ* gene of *Thermus thermophilus* HB8 (ATTC27634) (Terada et al., 1999), but optimized for expression in Bacillus by modification of the overall G+C% and codon usage.

Construct

The gene was put in a pBHA1 derived vector – pBHA1 is a derivate of the endogenous Bacillus plasmid pUB110 (see Annex II.E.1-1) - in the following way:

First a modified copy of the *malQ* gene was synthesized by annealing of complementary poly nucleotides to produce double stranded DNA fragments and subsequent ligation of

these products using short complementary ends. The polynucleotides were designed so as to contain optimal codons for each amino acid, based on the preferred codon usage in *Bacillus subtilis* (da Rocha et al., 1999), but also applicable to other related Bacilli such as *Bacillus amyloliquefaciens*. In this way a gene was obtained encoding the same primary amino acid sequence as the wild-type *T. thermophilus* strain but optimized for translation in its production host. The gene, designated masQ, was synthesized as two separate fragments, the first covering the 5' end of the gene (841 basepairs), the second covering the 3' terminus (689 basepairs). These two fragments each were sequentially cloned into plasmid pBHA12. Plasmid pBHA12 is a derivative of plasmid pBHA1 but containing a promoter derived from the amyQ gene of *Bacillus amyloliquefaciens* for high-level expression. The sequential cloning of the fragments yielded the vectors pGBMAS3 and pGBMAS4 respectively.

Vector pGBMAS4 was digested with BamHI and the fragment containing the masQ sequences were religated. This ligation mixture was used to transform naturally competent cells of B. subtilis 1A40 and the resulting transformants were selected for their plasmid borne resistance to the antibiotic kanamycin or neomycin. This yielded the vector pGBB05MAS1, in which the reading frame of the masQ gene was resored by fusion of the 5'and 3' fragments and the entire E. coli moiety of the parental vector was removed. The construction and the sequence of the final expression vector is schematically shown in Annex II.E.1-6.

Transformation and isolation of the recombinant strain

The vector pGBB05MAS1 was finally transferred to Bacillus amyloliquefaciens EBA127 by electroporation.

From the kanamycin or neomycin resistant clones a strain with the desired amylomaltase activity was selected and designated as Bacillus amyloliquefaciens strain MAS-3.

F. Information related to the dietary exposure to the processing aid

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

DSM amylomaltase is proposed for use in the preparation of Etenia™, a fat and casein replacer.

As such, applications in foodstuffs can be (list is not extensive):

1) Yoghurt and Drink yoghurt
2) Cheese analogues
3) Low-fat spreads

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

Based on the information given in Section II.A.4, the following calculation can be made:

Final food	Enzyme use level in food ingredient	Amount of ingredient in final food	Residual amount of (inactivated) enzyme in final food	Amount of TOS in final food
yoghurt yoghurt drinks, low-fat spreads	3800 ATU/kg dry starch	1.5 – 5.5%	57 – 209 ATU/kg[3]	1.47 – 5.40 mg/kg

As mentioned in Section II.A.5, the amylomaltase is inactivated after the potato starch is modified. Therefore no amylomaltase activity could be determined in the final product Etenia™ and so in yogurt, cheese analogues, low fat spreads and etc.

Maximum amount of enzyme preparation to be used in each foodstuff

Generally, enzyme preparations are used in *Quantum Satis*.

In Etenia™, the recommended dosage of the amylomaltase is 3.8 units per gram of dry starch.

[3] Assuming that Etenia™ consists of 100% starch

Main reaction products and possible reaction products not considered normal constituents of the diet

Starch is a polymer of glucose molecules. These molecules are bound on the α-1,4 binding site and/or α-1,6 linkage. Native starch is a combination of amylose and amylopectin. Amylose does have mainly α-1,4 bonds which results in linear molecules of about 1000-6000 glucose units (Degree of Polymerization DP). Amylopectin does have additional α-1,6 bonds which results in branching of the molecules. The length of the branches is about DP 10-60.

Amylomaltase, the enzyme used for the production of Etenia™, breaks down α-1,4 linkages and in a second step catalyzes reactions with the formation of an other α-1,4 linkage. These results in two things: The amylose will be broken down and the length of the branches of the amylopectin will effectively increase. The side chain distribution of the amylopectin also will change, while these side chains will both be broken down and at the same time made longer by this enzyme.

This leads to the conclusion that Etenia™ is a special kind of potato starch which differs only in chain length distribution compared to potato starch and not in the primary structure and can thus be considered a normal constituent of the diet (Lichtenbelt and Van Uffelen, 2006)[4].

Since the enzyme is inactivated after the hydrolysis process, no further reaction products will be formed after that. The inactivated enzyme remains inert in the food as any other protein.

Possible effects on nutrients

Based on the information given above and in section II.A.3, there is no basis to believe that there will be any effect on nutrients apart from intentional effects on the amount of fat.

3. 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

The penetration percentage of amylomaltose is difficult to predict but from a company point of view we aim at a very high penetration percentage.

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

Estimated Daily Intake (EDI) in the Netherlands

The EDI was calculated based on the estimated consumption of Etenia™ used as fat replacer (Jansen et al., 2006). The 95th percentile for intake of Etenia™ is estimated to be 8.6-11.8 g/day. The highest intake is found in the children category 1-3 years; it results in an intake of 8.9 g/day for an 8 kg child, leading to an intake of 1.1 g/kg bw/day.

[4] Lichtenbelt and Van Uffelen use a previous name for Etenia™, namely Meltagel.

Based on the data given in Section II F.2, the following calculation can be made:

Residual amount of (inactivated) enzyme in final food (ATU/kg)	**Estimated daily intake (g food/kg bw/day)**	**Estimated daily intake of (inactivated) enzyme (ATU/kg bw)**
57 - 209	1.1	0.063 – 0.23

H. List of Annexes

I.7-1 Declaration of exclusive capturable commercial benefit

I.9-1 Statutory declaration

II.A.3-1 Analytical protocol for determination of amylomaltase activity

II.A.4-1 Flow diagram of manufacturing process

II.A.5-1 Certificates of analysis of amylomaltase

II.A.5-2 CHO-MTT Cytotoxicity screening test for Bacillus toxins. CRO Report No. DFS 001/031176.

II.C.2-1 14–days oral toxicity (range-finding) study

II.C.2-2 90–days oral toxicity study

II.C.2-3 Ames test

II.C.2-4 Chromosomal aberration test

II.D.1-1 Taxonomic determination of classical parent strains EBA-1 and BZ-53

II.D.1-2 Taxonomic determination of recombinant amylomaltase strain MAS-3

II.D.1-3 Genealogy of current amylomaltase production strain

II.D.2-1 JECFA safety evaluation of various enzymes produced by *Bacillus amyloliquefaciens (subtilis)*

II.D.2-2 Opinion of the Dutch Ministry of Environment about strain EBA-127 as suitable host for the construction of gmo's (in Dutch and in English)

II.D.2-3 Opinion of the Dutch Ministry of Environment about strain EBA-127 as GMO self-clone (in Dutch and in English)

II.D.2-4 Advice of the Dutch Ministry of Environment regarding the classification of the current production strain (in Dutch and English)

II.D.2-5 Advice of the French Genetic Committee regarding the classification of the current production strain (in French and English)

II.E.1-1 Detailed information about the plasmid pUB110

II.E.1-2 Physical map of the aprA deletion vector, pGBBdapr1

II.E.1-3 Schematic representation of the procedure for the construction of gene disruptions using the thermosensitive replicon of pE194-based vector

II.E.1-4 Southern hybridisation analyses of the integration/excision strains

II.E.1-5 Qualitative assessment of the protease deficiencies of EBA126 (and EBA127)

II.E.1-6 Construction and nucleotide sequence of vector pGBB05MAS-1

I. Literature

Arrêté. (2006) *Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires,* ECOC0600115A, available at: http://admi.net/jo/20061202/ECOC0600115A.html

Cummings H.J. (1996). Danone Chair Monograph of Institute of Danone, Belgium. The large intestine in Nutrition and Disease, available at: http://www.angelfire.com/folk/cusp/images/large_intestine.pdf

De Boer, A.S. and Diderichsen, B. (1991) *On the safety of Bacillus subtilis and B. amyloliquefaciens: a review,* Appl. Microbiol. Biotechnol. 36 (1) 1-4

Douglass, J.S., Barraj, L.M., Tennant, D.R., Long, W.R. and Chaisson, C.F. (1997) *Evaluation of the budget method for screening food additive intakes,* Food Additives & Contaminants: Part A 14 (8) 791-802

Jansen, M.C.J.F., Van den Bosch, L.M.C. and Hulshof, K.F.A.M. (2006) *Anticipated intake of starch ingredient and consequences for intake of dietary fat and fat-soluble vitamins.* TNO Report V6772

Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology Evaluation of allergenicity of genetically modified foods (2001). Evaluation of allergenicity of Genetically Modified Foods, available at: http://www.who.int/foodsafety/publications/biotech/en/ec_jan2001.pdf

Joint FAO/WHO Expert Committee on Food Additives, 67th meeting, Rome, 20-29 June 2006, *General Specifications and Considerations for Enzyme Preparations Used in Food Processing,* WHO Technical Report Series 940 (2006), Annex 5, 89-96, available at: http://www.who.int/ipcs/publications/jecfa/reports/trs940.pdf

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

Priest, F.G. et al. (1987) *Bacillus amyloliquefaciens* sp. nov., nom. rev. Int. J. Syst. *Bacteriol*. 37 69-71

Da Rocha, E.P.C. Danchin, A., and Viari, A. (1999) Translation in *Bacillus subtilis*: roles and trends of initiation and termination, insights from a genome analysis. A. *Nucleic Acids Res* 27: 3567-3576

Tafazoli S., Wong A.W., Akiayama T., Kajiura H., Tomioka E., Kojima I., Takata H., Kuriki T. (2010) Safety evaluation of amylomaltase form Thermus aquaticus, Regul. Toxicol. Pharmacol. 57 62-69

Terada Y, K. Fujii, T. Takaha, S. Okada (1999) *Thermus aquaticus* ATCC 33923 amylomatase gene cloning and expression and enzyme characterization: production of cycloamylose. *Appl Environm Microbiol* 65: 910-915

Underkofler, L.A., Barton, R.R. and Rennert, S.S. (1958) *Production of microbial enzymes and their applications,* Appl. Environ. Microbiol. 6 (3) 212

Vehmaanpera J, Steinborn G, Hofemeister J. (1991) Genetic manipulation of *Bacillus amyloliquefaciens*. *J Biotechnol*. 1991 19:221-40.

III APPLICATION CHECKLIST

General Requirements (3.1)	
■ Form of application ■ *Executive Summary* ■ *Relevant sections of part 3 identified* ■ *Pages sequentially numbered* ■ *Hard copies capable of being laid flat* ■ *Electronic and hard copies identical* ■ Applicant details ■ Purpose of the application	■ Justification for the application ■ Information to support the application ■ Assessment procedure ■ Confidential Commercial Information □ *Confidential material separated in both electronic and hard copy* ■ Exclusive Capturable Commercial Benefit ■ International standards ■ Statutory Declaration
Food Additives (3.3.1)	
Not applicable	
Processing Aids (3.3.2)	
■ Type of processing aid ■ Identification information ■ Chemical and physical properties ■ Manufacturing process ■ Specification information □ Industrial use information (chemical only) □ Information on use in other countries (chemical only) □ Toxicokinetics and metabolism information(chemical only) □ Toxicity information (chemical only) □ Safety assessments from international agencies (chemical only)	■ Information on enzyme use on other countries (enzyme only) ■ Toxicity information of enzyme (enzyme only) ■ Information on source organism (enzyme from micro-organism only) ■ Pathogenicity and toxicity of source microorganism (enzyme from micro-organism only) ■ Genetic stability of source organism (enzyme from micro-organism only) ■ Nature of genetic modification (PA from GM micro-organism only) ■ List of foods likely to contain the processing aid ■ Anticipated residue levels in foods ■ Percentage of food group to use processing aid ■ Information on residues in foods in other countries(if available)
Nutritive Substances (3.3.3)	
Not applicable	