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November 26, 2018

VIA FEDERAL EXPRESS

Susan Carlson, Ph.D
Director
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Re: GRAS Notification for Beta-Galactosidase From a Genetically Engineered Strain of *Kluyveromyces lactis*

Dear Dr. Carlson:

On behalf of DSM Food Specialties ("DSM"), we are submitting under cover of this letter, one paper copy of a generally recognized as safe ("GRAS") notification for a beta-galactosidase enzyme preparation derived from *Kluyveromyces lactis*. DSM has determined through scientific procedures that beta-galactosidase is GRAS for use in dairy products such as milk, milk powder, fermented milk products and yoghurt, fresh cheeses, e.q. quark, ice cream, milk based deserts, whey, infant and follow-on formula.

The enzyme preparation is added to the milk and other dairy products. The beta-galactosidase hydrolyzes terminal non-reducing beta-D-galactose residues in beta-D-glactosides. Beta-galatosides as substrates include lactose and various glycoproteins. The enzyme will be used in the production of dairy products to create lactose reduced milk and milk derived products. The enzyme is either heat denatured and inactivated during the pasteurization/sterilization step, or irreversibly denatured at low-PH in the environment of fermented milk products. The use of beta-galactosidase can thus be regarded as a processing aid because there is no function in the finished food.

Pursuant to the regulatory and scientific procedures established by 21 C.F.R. § 170.36, the use of beta-glucosidase from *Kluyveromyces lactis* is exempt from premarket approval requirements of the Federal, Food, Drug, and Cosmetic Act, because the notifier has determined that such use is GRAS.

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If you have any questions regarding this notification, or require additional information to aid in the review of DSM's conclusion, please do not hesitate to contact me via email at gary.yingling@morganlewis.com or by telephone, (202)739-5610.

Sincerely,

[redacted]

Gary L. Yingling

cc: DSM Food Specialties

GRAS NOTICE

BETA-GALACTOSIDASE FROM A GENETICALLY ENGINEERED STRAIN OF *KLUYVEROMYCES LACTIS*

November 2018

DSM Food Specialties
PO Box 1
2600 MA Delft
The Netherlands
Tel: 31 611377088

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1.0 SIGNED STATEMENTS AND CERTIFICATIONS

1.1. Submission of GRAS Notice

DSM Food Specialties (DSM) is hereby submitting a Generally Recognized as Safe (GRAS) notice in accordance with the provisions of 21 CFR part 170, subpart E.

1.2. Name and Address of Notifier

DSM Food Specialties
PO Box 1
2600 MA Delft
The Netherlands

1.3. Name of Notified Substance

The common or usual name of the notified substance is beta-galactosidase (lactase). It consists of beta-galactosidase produced by submerged fed-batch fermentation using a selected, pure culture of *Kluyveromyces lactis*; the strain used to produce beta-galactosidase is genetically engineered. The beta-galactosidase preparation is produced and sold in liquid form, standardized with glycerol.

1.4. Intended Conditions of Use

The beta-galactosidase preparation is intended for use in dairy products such as milk, milk powder, fermented milk products and yoghurt, fresh cheeses, e.g. quark, ice cream, milk-based desserts, whey, and milk-based infant and follow-on formula. The enzyme is either heat denatured and inactivated during the pasteurization/sterilization step, or irreversibly denatured at low-pH in the environment of fermented milk products. The use of beta-galactosidase can thus be regarded as a processing aid because it has no function in the finished food.

1.5. Statutory Basis for GRAS Conclusion

Pursuant to 21 C.F.R. 170.30, DSM has determined, through scientific procedures, that the beta-galactosidase (lactase) enzyme preparation from a genetically engineered strain of *Kluyveromyces lactis* is GRAS for use as an enzyme for the hydrolysis of lactose at levels not to exceed good manufacturing practices.

1.6. Exclusion from Premarket Approval Requirements

It is the view of DSM that the substance is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, based on the conclusion by DSM that the beta-galactosidase is GRAS under the conditions of its intended use in dairy processing. DSM

recognizes certain uses (*e.g.*, processing of milk for use in infant formula) may be subject to additional requirements under the Federal Food, Drug, and Cosmetic Act[1].

1.7. Availability of Information for FDA Review

The data and information that are the basis for DSM's GRAS determination are available for FDA's review, and copies will be provided to FDA upon request, in either electronic format or by paper copy. Requests for copies and arrangements for review of materials cited herein may be directed to:

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1.8. Exemptions from FOIA Disclosure

This notification does not contain confidential data or proprietary information, and therefore no FOIA exemptions are claimed.

1.9. Authorization to Share Trade Secrets with FSIS:

DSM does not anticipate that FSIS consultation will be required. However, should FDA find the need to share the information in this application with FSIS, DSM has no objections.

[1] Manufacturers of infant formula are required to register with and make a submission to U.S. FDA for any new infant formula, which includes any infant formula that has had a major change in its formulation or processing.

1.10. Certification

On behalf of DSM Food Specialties, I certify that, to the best of my knowledge, the GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to me and DSM Food Specialties, and pertinent to the evaluation of the safety and GRAS status of the beta-galactosidase (lactase) from a genetically engineered strain of *Kluyveromyces lactis* for use as a processing aid in the manufacture of dairy products, including infant and follow-on formula.

[redacted] 11/26/18

Gary L. Yingling[redacted] Date
Senior Counsel
Morgan, Lewis, and Bockius LLP

2.0 IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

2.1. Identity of Notified Substance

Systematic name:	beta-D-galactoside galactohydrolase
Other names:	lactase; beta-lactosidase; maxilact; hydrolact; beta-D-lactosidase
Accepted name:	beta-galactosidase, lactase
IUPAC/IUB Number:	EC 3.2.1.23

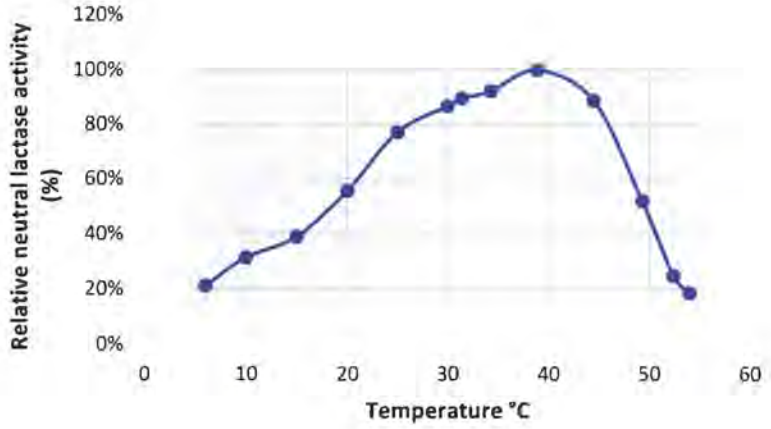
2.2. Principal Enzymatic Activity

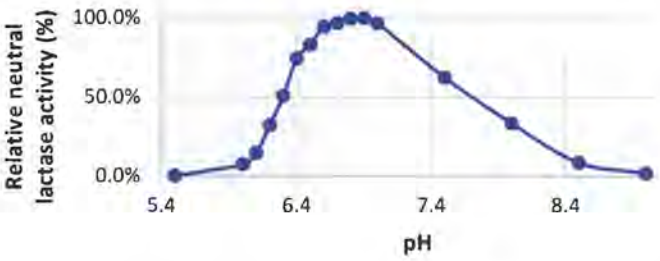
The beta-galactosidase hydrolyses beta-D-galactosides to release the terminal non-reducing beta-D-galactose residues. Beta-galactosides as substrates include lactose and various glycoproteins. The amino acid sequence of the beta-galactosidase protein described in this dossier differs in one amino acid when compared to the published amino acid sequence of beta-galactosidase from *Kluyveromyces lactis*[2]. Based on this sequence, it can be deduced that the Molecular Mass of ß-galactosidase protein is about 117.6 kDa

The activity of beta-galactosidase is expressed in Lactose Hydrolysis Units (LHU). One LHU is defined as the amount of enzyme that liberates 0.81 µmole glucose per minute using lactose as substrate under the conditions of the test (see Annex 1).

The activity of the beta-galactosidase described in this GRAS notice was measured under various pH and temperature conditions. The results are presented in the figures below.

[2] http://www.uniprot.org/uniprot/P00723





As can be concluded from the figures above, the food enzyme beta-galactosidase from the genetically engineered *Kluyveromyces lactis* strain KLA exhibits an optimal activity around pH 6.5-7.0 whereas the optimum temperature range lays between 35 and 45 °C. No enzyme activity is left at temperatures above 70 °C.

2.3. Specifications for Food Grade Material

The common starting material for all formulations is the ultra-filtration (UF) concentrate. Typically, its composition falls within the following ranges:

Enzyme activity	19,000- 25,000 LHU/g
Water (%)	70-80
Ash (%)	0-2
Proteins (%)	10-20

Apart from the enzyme protein in question, microbial food enzymes also contain some substances derived from the producing microorganism and the fermentation medium. These constituents consist of organic material (proteins, peptides, amino acids, carbohydrates, lipids) and inorganic salts.

In order to obtain a final formulation, the stabilized food enzyme is formulated either as a dry or a liquid preparation, depending on the final application in which the enzyme is intended to be used.

The Total Organic Solids (TOS, calculated as dry matter minus ash) and the activity/TOS ratio of the beta-galactosidase preparation were determined for 3 different batches and are included in the table below.

Batch number	Water (%)	Ash (%)	TOS (%)	Activity (LHU/g)	LHU/mg TOS
[redacted]	75.1	1.22	23.7	24,750	104.4
[redacted]	78.7	1.18	20.1	19,725	98.1
[redacted]	79.4	1.12	19.5	21,400	109.7
Mean					104.1

The finished product is subjected to extensive controls and complies with JECFA and FCC specifications:

Parameter	**Norm**
Lead	≤ 5 mg/kg
Coliforms	≤ 30 CFU/g
Salmonella	0 per 25g
Escherichia coli	0 per 25g
Antimicrobial activity	Absent by test
Mycotoxins	Not applicable for yeast enzymes

2.4. Description of the Production Organism

2.4.1. Name and designation

The strain used for the production of the beta-galactosidase enzyme preparations belongs to the species *Kluyveromyces lactis*.

2.4.2. Source of the organism

The current microorganism used for production of beta-galactosidase is a genetically engineered *Kluyveromyces lactis* strain, KLA.

Taxonomic identification of the *Kluyveromyces lactis* strain KLA (DS 79419) was done by means of genomic sequencing. The genomic sequencing was performed by BaseClear B.V. (Leiden, The Netherlands) using Illumina paired-end sequencing technology (average paired read length 125 bp, average fragment size ~250 bp). The 10.6 Mb long genome has been covered on average 240 times, with an average error rate of 0.0001 per base. The filtered paired-end

reads were used for an alignment with the *Kluyveromyces lactis* reference genome sequence of strain NRRL Y-1140 (Dujon et al., 2004, http://www.genolevures.org/).

The sequencing data demonstrated that the *Kluyveromyces lactis* strain KLA is closely related to *Kluyveromyces lactis* NRRL Y-1140.

2.4.3. **Strain improvement**

The parental strain DS 00332 was isolated from cheese, identified as *Kluyveromyces lactis*, and deposited as CBS 683 at the Centraalbureau voor Schimmelcultures in Utrecht, the Netherlands (now the Westerdijk Fungal Biodiversity Institute). This strain is a diploid, and has been used for more than 50 years for the production of beta-galactosidase (lactase) at DSM, initially at Societé Rapidase in Seclin (France), which was later on acquired by Gist-brocades which, in turn, was acquired by DSM. The recipient strain DS 38549 has been derived from DS 00332 by a series of classical strain improvements and single colony isolations.

The genetic modifications concern the integration of multiple expression cassettes of the modified beta-galactosidase gene, derived from *Kluyveromyces lactis* itself, at defined locations into the genome of the *Kluyveromyces lactis* recipient strain DS 38549. The variant β-galactosidase expression cassettes are inserted at two defined locations in the *Kluyveromyces lactis* genome, i.e. at the arylsulfatase (ARS) and β-galactosidase (LAC4) loci). The variant *Kluyveromyces lactis* beta-galactosidase has one amino acid substitution as compared to the published beta-galactosidase amino acid sequence[3]. This substitution was made in order to increase the efficiency of beta-galactosidase to hydrolyze lactose. The ß-galactosidase gene sequences were codon optimized for high-level expression in *Kluyveromyces lactis*, and synthesized chemically.

Furthermore, the genes coding for the native beta-galactosidase, invertase and arylsulfatase were deleted. The DNA fragments transformed to *Kluyveromyces lactis* for gene deletion and insertion contain markers that confer resistance to three different antibiotics. These antimicrobial resistance genes were all flanked by *lox* sites that allow precise excision by Cre recombinase. An episomal plasmid was used for the expression of Cre recombinase.

Genomic sequencing of the final strain confirmed the deletion of the arylsulfatase, invertase and native beta-galactosidase genes, and the integration of seven copies of the variant beta-galactosidase at the targeted integration sites. No ectopic integration was found. Furthermore, genomic sequencing confirmed the absence of any selection markers and vector sequences.

2.4.4. **The taxonomy**

The formal classification of *Kluyveromyces lactis* strain KLA is as follows:

Kingdom:	Yeast
Genus:	*Kluyveromyces*
Species:	*Kluyveromyces lactis*

[3] http://www.uniprot.org/uniprot/P00723

Strain: KLA

2.4.5. **Stability of the Transformed Genetic Sequence**

The phenotypic stability of the *Kluyveromyces lactis* strain KLA is proven by its capacity to produce a constant level of the beta-galactosidase enzyme. This was assessed by measuring the enzyme activity in relation to the TOS content in three independent batches of the food enzyme, as mentioned in section 2.3 above. The information in section 2.3 confirms the stability of the phenotype of the *Kluyveromyces lactis* strain KLA with respect to the production of the desired enzyme.

The genotypic stability of the *Kluyveromyces lactis* strain KLA was assessed by comparing the genotype of (i) the original working cell bank (WCB) of the GMM strain KLA that was used as inoculation material for the fermentations, and (ii) samples at the end of fermentation (EOF) from three independent batches, i.e. [redacted]
Genetic stability was assessed by analysing five characteristic and unique fragments from the beta-galactosidase expression modules (two separate fragments from the ARS locus, and three separate fragments from the LAC4 locus). Detection of the specific fragments was performed by PCR.

The results show that no changes in the genetic structure of the beta-galactosidase expression cassettes are observed between the WCB (start of fermentation) and the *Kluyveromyces lactis* KLA strain at the end of the fermentation. These data thus confirm the genetic stability of the strain.

2.4.6. **Nature of pathogenicity and virulence, infectivity, toxicity and vectors of disease transmission**

Kluyveromyces lactis is globally regarded as a safe microorganism:

- In the USA, *Kluyveromyces lactis* is not listed in the National Institutes of Health Guidelines for Research involving Recombinant or Synthetic Nucleic Acid Molecules (NIH Guidelines; USA, 2016). It therefore qualifies as a Risk Group 1 organism, provided that a risk assessment is conducted based on the known and potential properties of the organism and its relationship to organisms that are listed as Risk Group 2, 3 or 4 in the NIH Guidelines. Risk Group 1 microorganisms, such as baker's yeast, have the lowest safety concerns.

- In Europe, *Kluyveromyces lactis* is classified as a low-risk-class microorganism, as exemplified in the listing as Risk Group 1 in the microorganism classification lists of the German Federal Office of Consumer Protection and Food Safety (Germany, 2013), the Swiss Agency for the Environment, Forests and Landscape (Switzerland, 2004), and the Dutch Commission on Genetic Modification (Netherlands, 2011). It is not mentioned on the list of pathogens on the Belgian Biosafety Server (Belgium, 2010).

- In the EU, *Kluyveromyces lactis* does not appear on the list of pathogens in Annex III of Directive 2000/54/EC (EU, 2000) on the protection of workers from risks related to exposure to biological agents at work.

- The European Food Safety Agency (EFSA) maintains a list of the biological agents to which the Qualified Presumption of Safety (QPS) assessment can be applied. In 2007, the Scientific Committee set out the overall approach to be followed and established the first list of biological agents. The QPS list is reviewed and updated annually by the Panel on Biological Hazards (BIOHAZ). The safety of *Kluyveromyces lactis* as a production organism has been assessed by EFSA, and it has been accorded QPS status (EFSA, 2007, 2018).

- Finally, *Kluyveromyces lactis* has been included in the 2002 "Inventory of microorganisms with a documented history of use in food", prepared by the International Dairy Federation (IDF) in collaboration with the European Food and Feed Cultures Association (EFFCA; Mogensen et al., 2002a, b), as well as in the 2011 update on microbial food cultures for beneficial use (Bourdichon et al., 2012).

As a result, *Kluyveromyces lactis* can be used at the least stringent containment level at large scale, GILSP, as defined by OECD (1992).

Thorough literature searches have been done on the pathogenicity of the production microorganism, *Kluyveromyces lactis*, which provided very little evidence for potential concern. On rare occasions, *Kluyveromyces* species have been suggested to act as opportunistic pathogens in humans (Lutwick et al., 1980; Corpus et al., 2004; Reuter et al., 2005; Gomez-Lopez et al., 2010). The majority of these cases were due to *Kluyveromyces marxianus*, and only solitary cases to *Kluyveromyces lactis*. Typically, the patients affected were immuno-compromised and/or catheterized (e.g., Lutwick et al., 1980; Corpus et al., 2004; Reuter et al., 2005), and the *Kluyveromyces* isolates proved to be highly susceptible *in vitro* to currently licensed antifungal compounds (Gomez-Lopez et al., 2010). In line with the very low frequency of opportunistic infections, *Kluyveromyces* is not included in authoritative lists of emerging yeast pathogens (Hazen, 1995; Arendrup et al., 2013).

Thus, there are absolutely no indications that would compromise the statement that the yeast *Kluyveromyces lactis* is non-pathogenic for (immuno-competent) humans or animals.

2.4.7. **Natural habitats, geographic distribution and climatic characteristics of the original habitats**

Kluyveromyces lactis (also called "dairy" or "milk yeast") is a natural and indispensable component of dairy culture processes where lactose concentration is high. Wild-type *Kluyveromyces lactis* isolates have mostly been isolated from dairy processes such as yogurt, cheese and buttermilk making.

Kluyveromyces lactis is a budding yeast, similar to baker's yeast, *Saccharomyces cerevisiae* (Schaffrath and Breunig, 2000; New England Biolabs, 2010). Both organisms are

morphologically similar, with *Kluyveromyces lactis* cells generally being smaller than *Saccharomyces cerevisiae* cells. Both organisms can exist in either a haploid or a diploid state. Diploids of either organism can undergo sexual reproduction and generate four haploid ascospores, the products of meiotic segregation. Similar to *Saccharomyces cerevisiae*, *Kluyveromyces lactis* haploids can have either "a" or "α" mating types. No other modes of genetic transmission have been described for *Kluyveromyces lactis.*

In nature, *Kluyveromyces lactis* possesses a highly expressed beta-galactosidase that degrades milk sugar (lactose) to galactose and glucose. Therefore, *Kluyveromyces lactis* can grow on lactose as sole carbon source. This property can be used to select *Kluyveromyces lactis* isolates from dairy products. Additionally, *Kluyveromyces lactis* is Crabtree-negative and does not produce ethanol aerobically.

It is of relevance to note that enzymes that are produced by *Kluyveromyces lactis* have been approved e.g. in France and Denmark. *Kluyveromyces lactis* has been used for industrial production for more than 50 years (Bonekamp & Oosterom, 1994). In the US, Olempska-Beer et al. (2006) from the FDA have summarized the safety of genetically engineered microorganisms, including *Kluyveromyces lactis*, used as hosts for enzyme-encoding genes. The safety of *Kluyveromyces lactis* is well-documented. This species has been used for many years as a source of beta-galactosidase used for conversion of lactose to galactose and glucose in milk and milk products. The beta-galactosidase enzyme preparation from *Kluyveromyces lactis* was affirmed by FDA as GRAS (21 CFR 184.1388) in 1984. Another enzyme preparation, chymosin, from a genetically engineered strain of *Kluyveromyces lactis*, was affirmed by FDA as GRAS (21 CFR 184.1685) in 1993.

2.4.8. **Good Industrial Large Scale Practice GILSP**

The parental strain, *Kluyveromyces lactis* DS 00332, has been used for more than 50 years for the production of beta-galactosidase (lactase) at DSM in Seclin, France. No adverse effects on the environment or health of the personnel employed in the fermentation facilities have been observed.

The organism is non-pathogenic, does not produce adventitious agents and has an extended history of safe industrial use. Therefore, the beta-galactosidase production organism is considered to be of low risk and can be produced with proper controls and containment procedures in large-scale production. This is the concept of Good Industrial Large Scale Practice (GILSP), as endorsed by the Organization of Economic Cooperation and Development (OECD). In the facilities of DSM Food Specialties for the large-scale production of food enzyme products, only fermentations not exceeding the GILSP level of physical containment are carried out.

2.4.9. **Absence of Toxins**

Kluyveromyces lactis is commonly named "dairy" or "milk yeast" and does not produce mycotoxins. Mycotoxin analysis is done routinely on all the enzyme preparations from the production site. Therefore, the absence of toxins is assured in any enzyme preparation produced by *Kluyveromyces lactis*.

2.5. Method of Manufacture

2.5.1 Overview

Beta-galactosidase is produced by DSM in a controlled submerged fed-batch fermentation of a selected, pure culture of *Kluyveromyces lactis* (see Section 2.4). The production process includes the fermentation, recovery (downstream processing) and formulation of the product. An overview of the different steps involved is given in Annex 2.

2.5.2 Raw Materials

The raw materials used for the fermentation and recovery of the product are suited for the intended use leading to the required safety status of the product. This is confirmed by the toxicological studies performed (see Section 6.4 of this dossier). The raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of DSM. The raw materials used for the formulation are of food grade quality.

The fermentation and feed media used have been developed for optimum production of enzymes (in this case beta-galactosidase) by the *Kluyveromyces lactis* production organism.

2.5.3 Fermentation Process

Beta-galactosidase is produced by DSM in a controlled submerged fed-batch fermentation of a pure culture of *Kluyveromyces lactis*. All equipment is carefully designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms.

The fermentation process consists of three steps: inoculum, followed by the seed fermentation and the main fermentation. The whole process is performed in accordance with Good Food Manufacturing Practice.

Biosynthesis of beta-galactosidase occurs during the main fermentation. To produce the enzyme of interest, a submerged, aerobic fed-batch fermentation process is employed, using a stirred tank fermenter. To control the growth of the production organism and the enzyme production, the feeding rate of the feed medium is based:

- upon a predetermined profile; or
- on deviation from defined set points for pH or dissolved oxygen concentration.

Growth of the production organism and the level of enzyme production are checked at the end of the main fermentation by analysis of aseptically collected samples.

2.5.4 Recovery Process

During fermentation, the enzyme protein accumulates inside the cells of the producing microorganism, *Kluyveromyces lactis*. Consequently, at the end of the fermentation step, the cells of the production organism, *Kluyveromyces lactis*, are lysed to release the enzyme protein into the fermentation medium which is done by treatment of the fermentation broth with a lytic agent. The amount of lytic agent used and residues that can be present in the final enzyme preparation are safe both for the general population and for infants. This treatment effectively kills the *Kluyveromyces lactis* cells. The cell material is separated from the enzymes by means of a simple membrane filtration process. Subsequently, the remaining particles are removed with a polish filtration and a germ filtration, and the enzyme solution then concentrated by ultrafiltration (UF).

2.5.5 Formulation and Standardization Process

To obtain a liquid enzyme preparation, the ultrafiltration (UF) concentrate is standardized with glycerol to the desired final enzyme activity.

2.5.6 Quality Control of Finished Product

The final beta-galactosidase preparation from the genetically engineered *Kluyveromyces lactis* strain KLA is analyzed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO in 2006 and the FCC (10^{th} edition). These specifications are described in Section 2.6.

2.6. General Production Controls (Good Manufacturing Practice)

Commercial demands require a strictly controlled production process.

The enzyme manufacturing site in France, is in operation since 1922 and has received ISO 9001-2000 certification.

Optionally, the fermentation and recovery steps of the beta-galactosidase manufacturing process can be outsourced to other manufacturing facilities located in the major industrial markets provided that they operate in compliance with GMP standards, HACCP principles and have FSSC certification.

2.6.1. Technical Measures

The batches of **primary seed material** are prepared, preserved, and stored in such a way that contamination and degeneration are avoided and genetic stability is secured. The vials are clearly labeled, and strict aseptic techniques are applied during the recovery of the culture.

Only sterilized **raw materials** are used to prepare the nutrient medium for the fermentation.

The **fermenter** is a contained system. Only sterilized air is used in the fermentation. Membrane valves, air filters and seals are regularly checked, cleaned, and replaced if necessary. Prior to inoculation, the fermenter is cleaned, rinsed, and sterilized. The sterilized nutrient medium and the complete biomass broth are transferred aseptically to the main fermenter. The methods used effectively prevent microbial contamination during fermentation.

The preparation of sterile media and the cleaning of the equipment are laid down in Quality Assurance documents and strictly followed.

Microbial contamination is prevented during downstream processing by several germ filtration steps. The filters are thoroughly cleaned for each production run.

2.6.2. **Control Measures**

After preparation of a new batch of **primary seed material,** samples are checked for identity, viability, and microbial purity. If these parameters meet the internal specifications, the strain is tested for production capacity. Only if the productivity and the product quality meet the required standards, the new batch of primary seed material will be accepted for further production runs.

The **raw materials** used for the fermentation and recovery of the product are suited for the intended use leading to the required safety status of the product. The raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of DSM. The raw materials used for the formulation are of food grade quality.

At regular intervals during the **seed fermentation** samples are taken aseptically for analysis of pH and microbiological quality.

During the **main fermentation** the dissolved oxygen content, pH, temperature, viscosity and microbial quality are monitored. If microbial controls show that significant contamination has occurred, the fermentation will be discontinued.

Also during **downstream processing** samples are being taken and checked for the level of microbial contamination. If these checks show that significant contamination has occurred, the downstream processing will be discontinued.

The consistency of the manufacturing process was confirmed by analytical results obtained from consecutive 3 batches data in the table below. This conforms to the current edition of USP FCC monograph, and to JECFA specifications.

Batch number	Lead mg/kg	*Salmonella* per 25g	Coliforms CFU/g	*Escherichia coli* per 25g	Antimicrobial activity
[redacted]	0.01	absent	10	absent	not detected
[redacted]	0.02	absent	10	absent	not detected
[redacted]	0.01	absent	10	absent	not detected

3.0 DIETARY EXPOSURE

3.1. Estimated Dietary Intake

It is important to note that DSM's beta-galactosidase produced by *Kluyveromyces lactis* strain KLA represents another type of lactase that will have to compete in essentially the same markets and foods as lactases currently in use. As a replacement for other lactases currently in use for dairy processing, DSM's enzyme would not be expected to substantially increase background intakes of this enzyme from current food uses.

Based on the intended applications and proposed maximum use levels, the following estimation of the human consumption can be made:

Application	Raw material (RM)	Maximal recommended use levels (mg TOS/kg RM)	Final food	Ratio RM/final food	Maximal level in final food (mg TOS/kg food)
Dairy processing	Milk	130	milk, hydrolysed milk, Dulce de leche, ice creams, milk derived desserts, fermented milk products (yogurt, sour cream, buttermilk, kefir, fermented dairy specialty)	1[4]	**130**

[4] It is assumed that 1 liter of milk is needed to produce 1 kilogram of fermented milk products. Therefore, a ratio RM/FF of 1 is taken into account.

Infant and follow-on formula	Milk	36	Infant and follow-on formula	1	**36**
Whey processing (solid food)	Whey	130	Baked products, confectionary, cereal bars	2.4[5]	**312**
Whey processing (beverages)	Whey	130	Soft drinks	0.9[6]	**117**

The Estimated Daily Intake (EDI) for both the general population aged 2 and older and infants will be calculated on the maximal level of the enzyme preparation, expressed in TOS, present in the final food.

In order to calculate the EDI for the general population the maximum dose levels and food consumption data for US people were taken into account. The NHANES database of 2011-2012 was used to assess the human consumption data of dairy products and baked goods (NHANES, 2011-2012). This database does not include dietary intake of beverages, and therefore the intake of soft drinks was based on consumption data from the USDA's 1994 and 1995 survey (Wilson, 1997). The consumption data is reported for individuals 2 years and over, based on the mean daily food consumption. In order to account for 'high-level' consumers, the 90th percentile of the intake level is taken to represent long-term daily intake estimates. The 90th percentile is approximately 2 times the mean intake level (US Food and Drug Administration, 2006).

The calorie intake per kilogram bodyweight is highest in the first weeks of life (Institute of Medicine, 2005). The first months an infant is expected to be either exclusively breastfed or exclusively formula fed. After 4-6 months, milk will no longer be the sole energy source as other foods will be consumed as well. A worst-case exposure calculation based on infants exclusively formula fed will therefore also cover infants and toddlers who consume both formula and other food sources.

The NHANES database includes data from individuals 2 years and over, as breast-fed children were excluded because breast milk was not quantified in dietary recall interviews. The EDI for infants can therefore not be calculated based on consumption data from the NHANES database and will be calculated separately.

In order to calculate the EDI for infants, consumption levels of milk reported by the Institute of Medicine was taken into account. In order to be conservative, the 95th percentile was used as a worst-case exposure scenario instead of the average consumption for infants. The 95th percentile can be estimated by adding two times the standard deviation to the average (Altman and Bland, 2005).

[5] Whey contains about 5% lactose. Typically, baked products contain up to 40% of whey products consisting of 30% lactose. Therefore, a ratio RM/FF of (1/50 / 1/300 x 0.4 =) 2.4 is taken into account.
[6] Whey contains about 5% lactose. Typically, beverages contain up to 15% of whey products consisting of 30% lactose. Therefore a ratio RM/FF of (1/50 / 1/300 x 0.15 =) 0.9 is taken into account.

3.1.1. EDI for the general population aged 2 and older

The NHANES database of 2011-2012 was used to assess the human (aged 2 and older) consumption data of dairy products and baked goods (NHANES, 2011-2012).

In the NHANES database the category 'total dairy' includes fluid milk, cheese, yogurt, and miscellaneous dairy. Therefore, the total dairy intake minus the cheese intake level was taken into account for the dairy processing application.

The total dairy intake, without considering cheese, for males and females 2 years and older is 0.99 cups/person/day, which equals 243 gram/person/day[7]. Assuming a body weight of 60 kg, the mean intake would be 4.05 g/kg bw/day. This consumption data is per individual, based on the mean daily food consumption. In order to account for high consumption, the 90th percentile of the intake level is taken. The 90th percentile is approximately 2 times the mean intake level (US Food and Drug Administration, 2006), resulting in a 90th percentile intake of 8.1 g/kg bw/day.

For baked products, the total grain consumption for males and females 2 years and older is 6.81 ounces/person/day, which equals 193 g/person/day. Based on a person weighing 60 kg, the mean intake would be 3.2 g/kg bw/day and the 90th percentile would be 6.4 g/kg bw/day.

For soft drinks, the intake of beverages from the USDA's 1994 and 1995 survey data was used (Wilson, 1997). The total consumption of 'fruit drinks and ades' was reported to be 92 gram/person and for carbonated soft drinks this was 327 g/person, leading to a total of 419 g/person/day. For a person with a body weight of 60 kg, the estimated mean and 90th percentile intake would be 2.5 g/kg bw/day and 5.0 g/kg bw/day, respectively.

Application	Maximal level in final food (mg TOS/kg food)	90th percentile intake level (g/kg bw/day)	Estimated daily intake of TOS (mg TOS/kg bw/day)
Dairy processing	130	8.1	$130 \times 14.6 \times 10^{-3} = 1.1$
Whey processing (solid food)	312	6.4	$312 \times 6.4 \times 10^{-3} = 2.0$
Whey processing (beverages)	117	5.0	$117 \times 5.0 \times 10^{-3} = 0.6$

Total **EDI for the general population** is 1.1 + 2.0 + 0.6 = **3.7 mg TOS/kg bw/day**.

3.1.2. EDI for infants

[7] 1 cup is equivalent to 245 gram https://www.ars.usda.gov/ARSUserFiles/80400530/pdf/fped/FPED_1314.pdf

In the Institute of Medicine report, in Table 5-18, it is indicated that infants 1 month of age consume 520 ± 131 kcal/d with an energy content of 0.68 ± 0.08 kcal/g (Institute of Medicine, 2005). The average consumption for infants 1 month of age is therefore (520 kcal/d / 0.68 kcal/g =) 765 g/d which corresponds to the average reference milk intake level of 0.78 L/day for infants ages 0 through 6 months suggested by the Institute of Medicine as well as to the mean milk consumption reported by the USDA's 1994 and 1995 survey data stating that mean total milk consumption is 775 grams/person for males and females below 1 year of age (Wilson, 1997).

In order to calculate a worst-case exposure, the 95th percentile was used instead of the average consumption. The 95th percentile can be estimated by adding two times the standard deviation to the average (Altman and Bland, 2005). The 95th percentile is [(520 + 2*131 kcal/d) / 0.68 kcal/g =] 1150 g/day.

A reference weight of 4.3 kg was used for infants of 1 month (based on 4.2 kg for girls and 4.4 kg for boys). This results in an estimated average intake level of 178 g infant formula/kg bw/day and the 95th percentile intake level of 267 g infant formula/kg bw/day.

The maximal level of TOS that could potentially end up in the infant formula is 36 mg TOS/kg. Taking into account the worst-case exposure scenario of 267 g infant formula/kg bw/day, the **EDI for infants is 9.6 mg TOS/kg bw/day**.

This EDI is based on consumption data of infants of 1 month old who are exclusively milk fed, as around this time the consumption per kilogram body weight is the highest (Institute of Medicine, 2005). Therefore, this worst-case exposure scenario will also cover formula fed infants and toddlers up to 2 years of age.

3.1.3. Interpretation of Estimated Dietary Intake

It should be stressed that the EDIs are based on conservative assumptions and represent highly exaggerated values because of the following reasons:

- It is assumed that ALL producers of the above mentioned foodstuffs and beverages use the specific enzyme beta-galactosidase from *Kluyveromyces lactis;*
- It is assumed that ALL producers apply the HIGHEST use level per application;
- It is assumed that the daily consumption of infant formula and of follow-on formula is based on the highest energy intake, which occurs only during the first 2 months from birth.
- It is assumed that the daily consumption of all food categories included (dairy, baked goods, and soft drinks) is always corresponding to the high-level consumers, which is certainly not the case as people who are high-level consumers of one food category are not likely to be also high-level consumers of other food categories.
- It is assumed that the amount of TOS does not decrease as a result of the food production process;

- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;

3.1.4. **Levels of Use**

Enzyme preparations are generally used in *quantum satis*. The average dosage of the enzyme depends on the type and quality of the raw materials used, and the process conditions. The levels of use expected to result in beneficial effects are described below.

Application	Final food	Maximal recommended use levels (mg TOS/kg RM)	Ratio RM/final food	Maximal level in final food (mg TOS/kg food)
Dairy processing	milk, hydrolysed milk, Dulce de leche, ice creams, milk derived desserts, fermented milk products (yogurt, sour cream, buttermilk, kefir, fermented dairy specialty)	130	1[8]	**130**
Infant and follow-on formula	Infant and follow-on formula	36	1	**36**
Whey processing (solid food)	Baked products, confectionary, cereal bars	130	2.4[9]	**312**
Whey processing (beverages)	Soft drinks	130	0.9[10]	**117**

The beta-galactosidase is added to the milk. The enzyme is either heat denatured and inactivated during the pasteurization/sterilization step, or irreversibly denatured at low-pH in the environment of fermented milk products. The beta-galactosides will not be functional in the final food.

3.1.5. **Purposes**

The beta-galactosidase hydrolyzes terminal non-reducing beta-D-galactose residues in beta-D-galactosides. Beta-galactosides as substrates include lactose and various glycoproteins. The enzyme may be used in the manufacture of a wide variety of dairy products containing lactose,

[8] It is assumed that 1 liter of milk is needed to produce 1 kilogram of fermented milk products. Therefore, a ratio RM/FF of 1 is taken into account.
[9] Whey contains about 5% lactose. Typically, baked products contain up to 40% of whey products consisting of 30% lactose. Therefore, a ratio RM/FF of (1/50 / 1/300 x 0.4 =) 2.4 is taken into account.
[10] Whey contains about 5% lactose. Typically, beverages contain up to 15% of whey products consisting of 30% lactose. Therefore a ratio RM/FF of (1/50 / 1/300 x 0.15 =) 0.9 is taken into account.

like milk, ice cream, Dulce de leche, hydrolyzed milk, fermented milk products like yogurt, sour cream, buttermilk, kefir, fermented dairy specialty, whey, or whey containing beverages.

In general, the technological need of the enzymatic conversion of beta-galactosides (lactose) with the help of beta-galactosidase can be described as:

- Removal of the milk sugar lactose to create lactose reduced milk and milk-derived products. These products are of benefit for lactose-intolerant individuals. Moreover, removal of lactose will minimize sandiness problems in certain milk products due to lactose crystals;

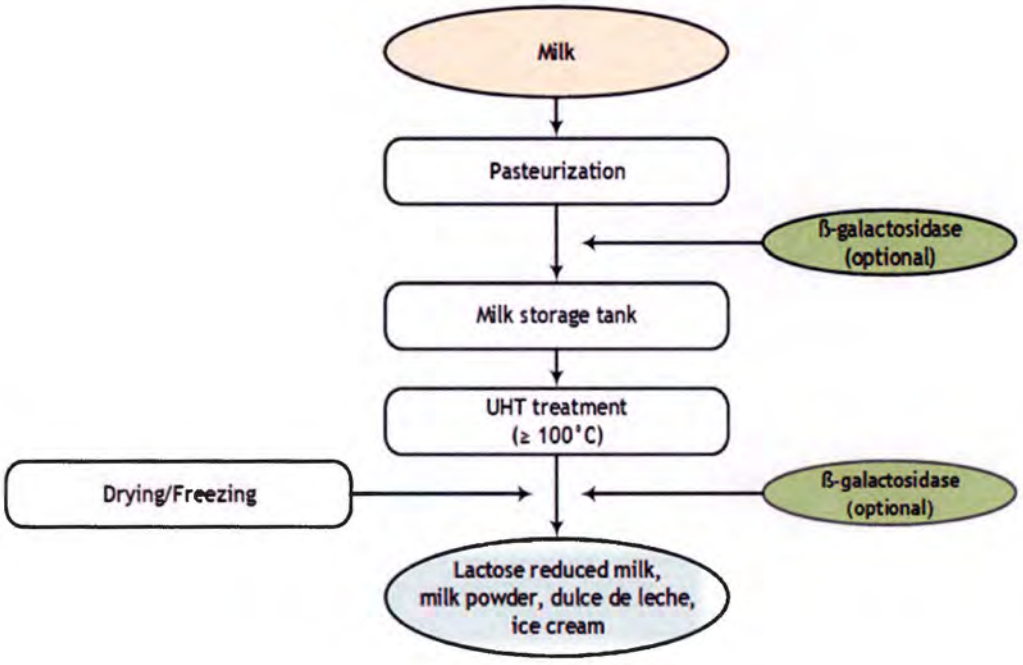
- Creation of the reaction products glucose and galactose, which possess a higher sweetness than lactose. Thus, treatment with beta-galactosidase will enhance the sweetness of milk and milk-derived products.

With the above technical benefits, DSM's intention is to use the beta-galactosidase from a genetically engineered strain of *Kluyveromyces lactis* in the manufacturing of dairy products.

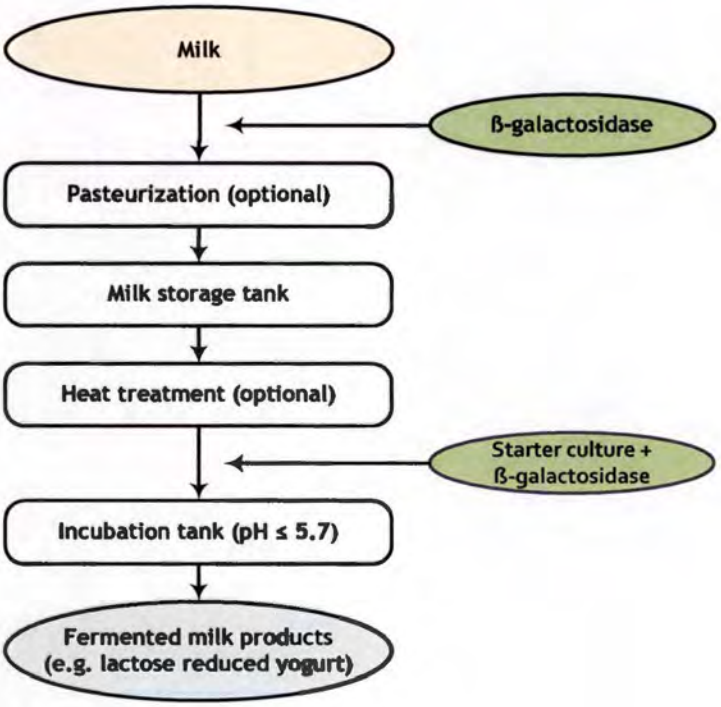
The beneficial effects mentioned in the various applications below are of value to the food chain because they lead to dairy products with reduced lactose content and improved digestibility for lactose intolerance population. Moreover, the applications lead to more effective production processes, resulting in better production economy and environmental benefits such as the use of less raw materials and the production of less waste.

The process steps of dairy products manufacture are shown below.

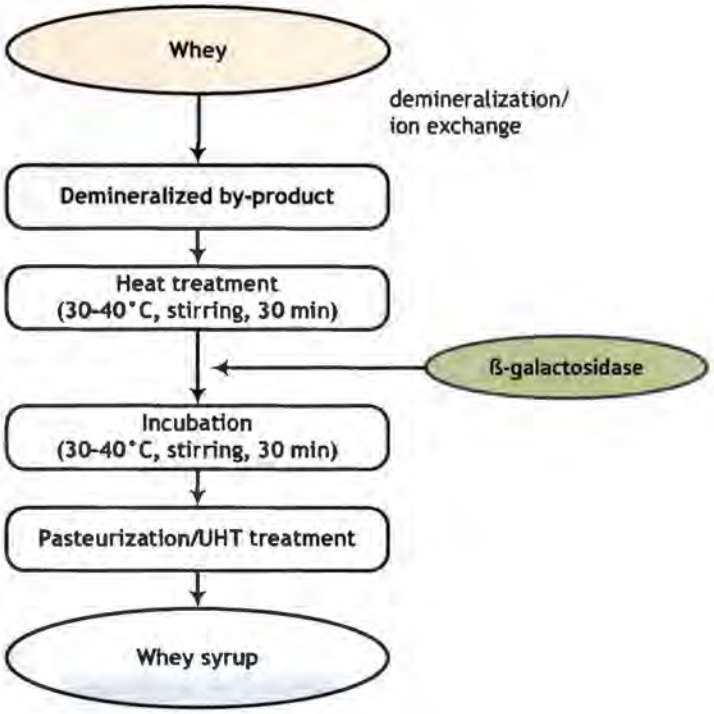
Milk hydrolysis process flow



Yogurt and fermented milk hydrolysis process flow



Whey hydrolysis process flow



3.2. Possible Effects on Nutrients

Beta-galactosidase acts, like any other enzyme, as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. It is not the enzyme protein itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function.

The substrate of the beta-galactosidase is lactose. Lactose is hydrolyzed to galactose and glucose by beta-galactosidase. Such enzyme activity is widely present in nature including humans as well as in food ingredients. The substrates and the reaction products are themselves present in foods. Consequently, no adverse effect on nutrients is expected.

3.3. Consumer Population

Milk is a significant dietary source of at least eight nutrients, including protein and calcium (Philips, and Briggs, 1975).

Milk also contains one carbohydrate (lactose), which is not well digested by certain populations. Lactose intolerant people cannot benefit from the nutritional benefits of milk and milk derived

products without having severe gastrointestinal (GI) discomfort (cramps, flatulence, and diarrhea). This so-called "lactose intolerance" is widespread within a substantial part of the global population.

In Europe, the average prevalence of lactose maldigestion is about 15%. Within Europe, prevalence varies from about 2-15% in the north-west to 85% in the south-east (Ingram et al., 2009; Leonardi et al., 2012). In the scientific opinion of EFSA related to lactase enzyme-related breakdown of lactose, the panel considers that breaking down lactose may be beneficial to the health of individuals with symptomatic lactose maldigestion (EFSA, 2009).

In the USA, the National Medical Association (NMA) has in 1999 urged the Department of Agriculture to promote lactose free dairy foods in both the Food Guide Pyramid and Dietary Guidelines for Americans (US NMA, 1999).

Milk and milk products are important sources of calcium in the diet. Women, who are lactose intolerant, may be at greater risk of osteoporosis resulting from low calcium-intake (Obermayer-Pietsch, 2004).

The milk which contains hydrolyzed lactose is therefore of special interest for the lactose intolerance population. The hydrolysis of lactose by beta-galactosidase reduces intolerance symptoms by breakdown of lactose into glucose and galactose. This also improves the availability of all other milk constituents like minerals, vitamins and proteins for lactose intolerant people.

The American Academy of Pediatrics Committee on Nutrition addressed the importance of lactose-free milk and dairy products when it published a review of lactose intolerance in infants, children and adolescents (Heyman, 2006). Infants who avoid milk and use alternatives e.g. soy or rice based formula may ingest less-than-recommended amounts of calcium needed for normal bone calcium accretion and bone mineralization. The lactose-free infant formula may be advantageous for the lactose intolerant infants. There are currently several lactose-free infant foods available on the USA market. It was also reported that symptoms in infant colic can be improved by reduction of lactose load with lactase (Kanabar, 2001). The American Academy of Family Physicians also notes that lactose-free formulas are an acceptable alternative to soy-based infant formula, for parents wishing to avoid lactose in infants with congenital lactase deficiency (O'Connor 2009).

In addition, the enzyme beta-galactosidase from *Kluyveromyces lactis* has a long history of use in food processing. As mentioned in section 2.4.7, a beta-galactosidase enzyme preparation from *Kluyveromyces lactis* was affirmed by FDA as GRAS (21 CFR 184.1388) in 1984.

As is shown in Section 3.1 of this dossier, the amount of enzyme TOS in the final food product is expected to be maximally 36-312 mg/ kg (0.0036-0.0312%).

Since the enzyme is present in food products at such low levels as an inactive protein, and because it is a naturally occurring substance in foods commonly ingested by humans, the consumer population will be unaffected by the presence of the enzyme preparation in food.

4.0 SELF-LIMITING LEVELS OF USE

Enzyme preparations are generally used in *quantum satis*. The average dosage of the enzyme depends on the type and quality of the raw materials used, and the process conditions. If using excessive amounts of enzyme, the food producer would experience untenable costs as well as negative technological consequences.

5.0 COMMON USE IN FOOD PRIOR TO 1958

DSM's GRAS conclusion is based on scientific procedures. It is important to note, however, that beta-galactosidase is a naturally occurring enzyme found in various microorganisms, plants, animals and humans where the enzyme is involved in lactose metabolism. In addition, beta-galactosidase has a long history of use in food processing. We believe that there is a strong argument for the use of beta-galactosidase prior to 1958 (as discussed in section 6.4), but we recognize that the genetically engineered strain of *K. lactis* was not available, and therefore this GRAS notice is based on scientific procedures.

6.0 SAFETY NARRATIVE

6.1. Overview

The beta-galactosidase (lactase) enzyme that is the subject of this GRAS notice is produced *via* controlled submerged fed-batch fermentation using a strain of *Kluyveromyces lactis* (KLA) developed by DSM. At the end of fermentation, cells are lysed to release the enzyme protein into the fermentation medium. The cell material is then separated from the enzyme by means of filtration, followed by concentration through ultrafiltration (UF). Glycerol is used to standardize the enzyme activity.

Kluyveromyces lactis strain KLA was genetically engineered to express a selected beta-galactosidase gene sequence from *Kluyveromyces lactis*. More details about the development of this production strain are provided below and in section 2.4 (and subsections thereof).

Confirmation of the KLA strain as *Kluyveromyces lactis* was made by BaseClear B.V. (Leiden, Netherlands) through genomic sequencing (see section 2.4.2). The sequencing data demonstrated that the *Kluyveromyces lactis* strain KLA is closely related to the well-characterized *Kluyveromyces lactis* strain NRRL Y-1140.

As discussed below and elsewhere in this dossier, in its assessment of the safety and GRAS status beta-galactosidase produced by *Kluyveromyces lactis* strain KLA, DSM considered the safety of the host organism (*e.g.*, history of safe use in food production); the nature and stability of the introduced (and/or deleted) genetic material; and the potential for production of harmful or toxic substances. The approach is consistent with the GRAS process for industrial microbial enzymes, described most recently by Sewalt *et al.* (2016) and also by Olempska-Beer et al. (2006).

In addition, the suitability of the beta-galactosidase enzyme preparation in food production is supported by the fact that the materials and methods used in the manufacture are tightly controlled and consistent with those generally used for the production of microbial enzymes for food use. The beta-galactosidase preparation meets the established specifications and the criteria in the USP FCC monograph on Enzyme Preparations, is free of mycotoxins, and a comparison of the amino acid sequence to known food allergens indicate it is not likely to produce any allergenic or sensitization reactions.

DSM considered that information on the safety of beta-galactosidase (lactase) from other safe and adequate microbial sources would be relevant to the safety assessment of beta-galactosidase produced by *Kluyveromyces lactis* KLA because, once separated from the production organism, these enzyme preparations would have comparable compositions and functions, i.e., consisting of cellular fractions containing beta-galactosidase (lactase) enzyme activity, residual amounts of processing aids used to separate the cellular fraction from production organism, and substances added as stabilizers or diluents.

With respect to the substitution of a single amino acid, DSM considered that the safety of the enzyme would not be affected because, as noted by Sewalt et al. (2016), such changes involve use of the same processes and cellular machinery as in wild-type enzymes, and are well within the natural variations of wild-type enzymes (Gabor et al. 2012).

6.2. Safety of the host organism

The safety of the production organism is paramount to assessing the probable degree of safety for enzyme preparations to be used in food production. According to the International Food Biotechnology Council, food or food ingredients are safe to consume if they have been produced according to current Good Manufacturing Practices from a nontoxigenic and nonpathogenic organism (Coulston and Kolbye, 1990a). A nontoxigenic organism is defined as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances" (Pariza and Foster, 1983).

As discussed previously, *Kluyveromyces lactis* (also called "dairy" or "milk yeast") is a natural and indispensable component of dairy culture processes where lactose concentration is high. Wild-type *Kluyveromyces lactis* isolates have mostly been isolated from dairy processes such as yogurt, cheese and buttermilk making.

Thorough literature searches have been done on the pathogenicity of the production microorganism, *Kluyveromyces lactis*, which provided very little evidence for potential concern. On rare occasions, *Kluyveromyces* species have been suggested to act as opportunistic pathogens in humans (Lutwick *et al.*, 1980; Corpus et al., 2004; Reuter *et al.*, 2005; Gomez-Lopez *et al.*, 2010). The majority of these cases were due to *Kluyveromyces marxianus*, and only solitary cases to *Kluyveromyces lactis*. Typically, the patients affected were immuno-compromised and/or catheterized (*e.g.*, Lutwick *et al.*, 1980; Corpus *et al.*, 2004; Reuter *et al.*, 2005), and the *Kluyveromyces* isolates proved to be highly susceptible *in vitro* to currently licensed antifungal compounds (Gomez-Lopez *et al.*, 2010). In line with the very low frequency of opportunistic infections, *Kluyveromyces* is not included in authoritative lists of emerging yeast pathogens (Hazen, 1995; Arendrup et al., 2013).

In a review of the safety of *Kluyveromyces lactis*, Bonekamp and Oosterom (1994) noted that *K. lactis* itself was reportedly consumed by humans in the form of an inactivated powder as a health food and protein supplement during the mid-1960s to early 70s, at up to 30 g per day, with no known problems.

In the *Federal Register* notice on the GRAS affirmation of lactase enzyme preparation from *Kluyveromyces lactis* (49 FR 47385-47386, Dec. 4, 1984), U.S. FDA refers to the same studies (Vrignaud, 1971; Gervais, 1973) reviewed by Bonekamp and Oosterom (1994), where subjects were fed 10 g or more per day of dried *K. lactis* as a dietary supplement in a variety of clinical situations, with no adverse health effects. FDA concluded that sufficient information was available to establish the safety of lactase enzyme preparation from *K. lactis*, based on evidence that the yeast *K. lactis* is safe, evidence that the materials used to make the enzyme preparation are safe, and information about the amount of exposure to this lactase enzyme preparation and to dried *K. lactis* yeast.

The safety of *Kluyveromyces lactis* as a production organism for beta-galactosidase (lactase) and as a host for recombinant strains is well-documented. Therefore, *Kluyveromyces lactis* would be considered a safe and appropriate host and donor organism, further supported by its classification as a low-risk organism in the U.S. and other countries (see section 2.4.6). In the EU, *Kluyveryomyces lactis* has Qualified Presumption of Safety (QPS) status (EFSA, 2007; 2018), meaning that the safety of this organism has been pre-assessed and confirmed by EFSA and that, accordingly, no toxicological data need to be submitted in the EU to establish the safety of this organism.

6.3. Safety of the Production Strain

The *Kluyveromyces lactis* KLA production strain was derived from a strain isolated from cheese (DS 00332) that has been used by DSM for more than 50 years for the production of beta-galactosidase (lactase). A series of classical strain improvements and selection steps were employed, along with genetic modifications that: (1) integrated multiple expression cassettes of the *Kluyveromyces lactis* beta-galactosidase gene at defined locations, with the synthesized gene sequences codon-optimized for high-level expression in *Kluyveromyces lactis*; (2) deleted genes coding for the native beta-galactosidase, invertase and arylsulfatase; and (3) resulted in a beta-galactosidase with one amino acid substitution compared to the native beta-galactosidase enzyme[11], which increases enzyme efficiency.

Genomic sequencing of the final production strain and comparison to the *Kluyveromyces lactis* reference genome confirmed the integration of seven copies of the variant beta-galactosidase at the targeted integration sites, no ectopic integration, deletion of the arylsulfatase, invertase and native beta-galactosidase genes, and absence of any selection markers and vector sequences.

The genotypic stability of *Kluyveromyces lactis* strain KLA was confirmed by comparing the genetic structure of the beta-galactosidase expression cassettes of the working cell bank (WCB) at the start of fermentation with samples at the end of fermentation. The ability of *Kluyveromyces lactis* strain KLA to produce a constant level of the beta-galactosidase enzyme provides evidence of its phenotypic stability (see section 2.3).

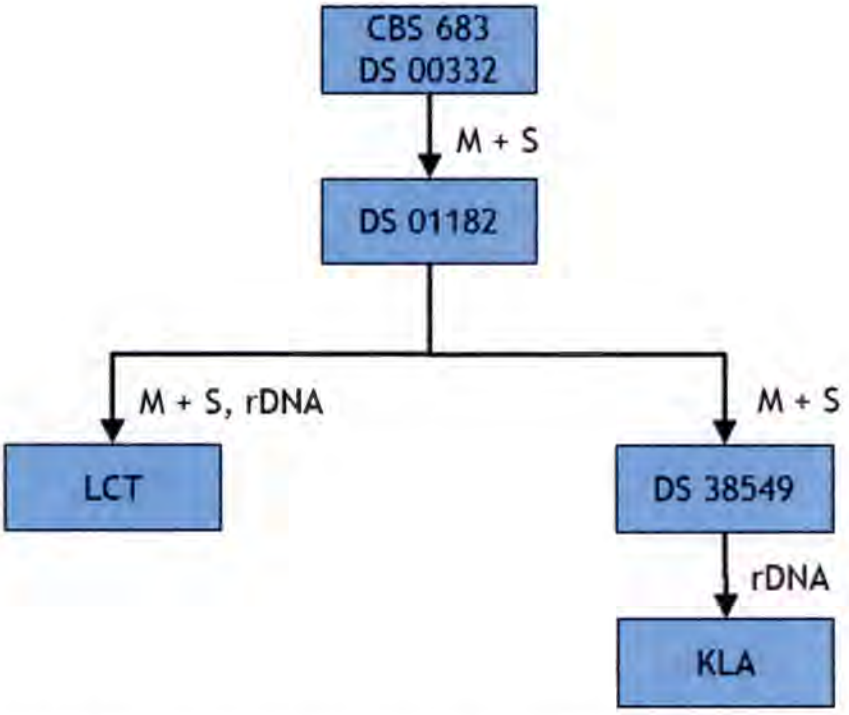
6.3.1. Safe strain lineage approach

DSM considered in its safety assessment that *Kluyveromyces lactis* KLA would qualify as an organism whose safety can be established based on the "safe strain lineage" approach, as supported by several publications (Pariza and Foster, 1983; Pariza and Johnson, 2001; and Pariza and Cook, 2010; Sewalt *et al.*, 2016). Specifically, DSM considered that the following conditions described by Pariza and Johnson (2001) would be met: (1) thorough characterization of the host organism as nonpathogenic and nontoxigenic; (2) determination of the safety of all new DNA that has been introduced into the host organism; and (3) use of modification procedure(s) appropriate for food use.

[11] http://www.uniprot.org/uniprot/P00723

This safe strain lineage approach is in line with the EFSA CEF guidelines (EFSA, 2009a, § 4.1.2), where it is mentioned that a full testing battery may be waived in specific cases. It has also been used in several GRAS notices that have generated no question from U.S. FDA (for *Aspergillus niger*: US FDA GRAS notification GRN 000183, GRN 000214, GRN 000296 and GRN 000345; for *Aspergillus oryzae*: US FDA GRAS notification GRN 000008, GRN 000034, GRN 000043, GRN 000075, GRN 000103, GRN 000106, GRN 000142 and GRN 000201; for *Trichoderma reesei*: US FDA GRAS notification GRN 000230, GRN 000315, GRN 000333 and GRN 000372). The safe strain lineage concept has also been the subset of at least one meeting between FDA and DSM.

Strains derived from the same strain lineage behave in the same way with respect to their growth requirements and metabolism. Consequently, food enzymes derived from the same strain lineage and produced under similar fermentation conditions are expected to have a comparable background of impurities that may originate from the production strains, and would thereby have comparable safety profiles. As such, toxicity testing of beta-galactosidase produced using *Kluyveromyces lactis* KLA was not considered necessary, because sufficient information is available otherwise (as specified above and elsewhere in this dossier) to establish a safe strain lineage, including the published safety studies described below (Coenen *et al.* 2000) of a beta-galactosidase enzyme produced by a different *Kluyveromyces lactis* strain (LCT) from this lineage, as shown in the figure below.



DSM's lineage of safe *Kluyveromyces lactis* production strains: DS ##### = deposition number in DSM's internal strain collection; M + S = mutation and selection; rDNA = use of recombinant DNA techniques; LCT = production strain for beta-galactosidase; KLA = a variant of beta-galactosidase (this dossier).

6.3.2. Toxicity studies of beta-galactosidase from a related *K. lactis* strain

Coenen *et al.* (2000) reported the findings of toxicity studies (genotoxicity and 28-day rat oral toxicity) of a beta-galactosidase produced using a different strain from this lineage, *Kluyveromyces lactis* LCT. The studies were carried out in accordance with OECD guidelines and in compliance with the principles of Good Laboratory Practice (GLP).

The Ames bacterial mutagenicity test showed no increase in the number of revertants in any of the five bacterial strains tested (S. *typhimurium* strains TA 98, TA 100, TA 1535 and TA 1537; *E. coli* strain, WP2*uvr*A), either in the presence or absence of S-9 mix up to 5 mg/mL of test substance. The *in vitro* chromosomal aberration test with human lymphocytes did not show biologically or statistically significant increases in the frequency of metaphases with aberrant chromosomes, with or without S-9 mix, up to 5 mg/mL test substance. It was therefore concluded that beta-galactosidase derived from *Kluyveromyces lactis* LCT was neither mutagenic nor clastogenic under the conditions employed.

Oral administration of this beta-galactosidase to rats for 28 days did not reveal noticeable signs of toxicity at any of the tested dosages. The No Observed Adverse Effect Level (NOAEL) was considered to be the highest concentration tested, equivalent to 1540 mg TOS/kg bw/day.

DSM considered that the NOAEL value of 1540 mg TOS/kg bw/day from the 28-day study above might serve as a useful reference for context with respect to consumer exposures, since the two enzymes are produced by organisms developed by DSM that have common ancestry, have a comparable amino acid sequence, and are produced under similar fermentation conditions, with comparable impurity profiles. We note, however, that the composition, function, and conditions of use of this beta-galactosidase enzyme preparation are consistent with those described previously, including the lactase enzyme preparation[12] derived from *Kluyveromyces lactis* that was affirmed as GRAS (49 FR at 47384, Dec. 4, 1984), for which FDA considered it impractical and unnecessary to delineate the levels of use (in the production of lactase-treated milk and lactose-reduced milk), due to variability in the conditions of use and the absence of any safety concerns.

6.4. Safety of the beta-galactosidase enzyme preparation

Beta-galactosidase is an enzyme naturally present in various microorganisms, plants, animals and humans. In addition, beta-galactosidase enzyme preparations from various microbial sources have a long history of safe use in food processing, as noted by Pariza and Foster (1983) and Pariza and Johnson (2001), further supported by the authorized uses in the U.S. and other countries, as noted below.

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[12] FDA describes the GRAS-affirmed lactase enzyme preparation from *K. lactis* as "a soluble enzyme preparation. It is composed of the cellular fraction of *K. lactis* that contains lactase enzyme activity, residual amounts of processing aids used to separate this cellular fraction from the yeast cells, and substances added to this cellular fraction as stabilizers or diluents. The lactase enzyme preparation contains the enzyme β-galactoside galactohydrase which catalyzes the hydrolysis of the disaccharide lactose to themonosaccharides glucose and galactose."

In the U.S., preparations affirmed as GRAS by FDA include lactase enzyme preparations from *Candida pseudotropicalis* (21 CFR 184.1387) and *Kluyveromyces lactis* (21 CFR 184.1388).

In its review of information supporting the GRAS affirmation of a lactase enzyme preparation derived from *Kluyveromyces lactis*, FDA noted that lactase enzyme preparations derived from *Kluyveromyces lactis* had been in use in the U.S. since the 1970s, including at the retail level for home use (49 FR at 47384, Dec. 4, 1984). On the other hand, in its response letter to GRAS notice GRN 000088, FDA noted that articles published during the 1950s reported use of lactase in the production of dairy products, *i.e.*, lactase from *Saccharomyces fragilis* (reclassified as *Kluyveromyces marxianus* = *Kluyveromyces lactis*) in milk products (Kirk and Othmer, 1957; Underkofler *et al.*, 1958), and lactase from other (or unspecified) yeasts in the production of ice cream (Reed, 1952; Underkofler and Ferracone, 1957).

A summary of other beta-galactosidase (lactase) preparations in GRAS notices submitted to U.S. FDA is provided in the table below.

GRN No.	Substance	Intended use	Date of closure	FDA's Letter
743	β-galactosidase from *Papiliotrema terrestris*	For use in the production of galacto-oligosaccharides	Pending (as of July 10, 2018)	Pending (as of July 10, 2018)
649	β-galactosidase enzyme preparation from *Bacillus circulans* produced in *Bacillus subtilis*	As an ingredient in foods	Nov 28, 2016	FDA has no questions
579	Lactase from *Bifidobacterium bifidum* produced in *Bacillus subtilis*	For use in the production of galacto-oligosaccharide for infant formula and in the production of fresh dairy products	Nov 5, 2015	FDA has no questions
572	Lactase from *Bifidobacterium bifidum* produced in *Bacillus licheniformis*	For use in the dairy industry to catalyze the hydrolysis of lactose in the production of lactose reduced products at levels not to exceed the minimum amount necessary to achieve the intended technical effect	Aug 28, 2015	FDA has no questions
510	Acid lactase from *Aspergillus oryzae* expressed in *Aspergillus niger*	For use as an enzyme in the hydrolysis of lactose in milk and whey	Sep 29, 2014	FDA has no questions
485	Beta-galactosidase enzyme preparation	As an ingredient in foods	Apr 15, 2014	FDA has no questions
132	Lactase enzyme preparation from *Aspergillus niger*	Use in the dairy industry to hydrolyze lactose found in milk, whey, cheese, yogurt, and other dairy products	Dec 12, 2003	FDA has no questions

88	Invertase enzyme preparation from *Saccharomyces cerevisiae* and lactase enzyme preparation from *Kluyveromyces marxianus*	Use in foods in general as an enzyme in accordance with current good manufacturing practices	Apr 3, 2002	FDA has no questions

As noted previously, the amino acid sequence of the beta-galactosidase protein described in this dossier differs by one amino acid when compared to the published amino acid sequence of beta-galactosidase from *Kluyveromyces lactis*[13]. A comparison to the amino acid sequence of other beta-galactosidases revealed the following: the variant beta-galactosidase described in this dossier has a single amino acid substitution compared to the *Kluyveromyces lactis* beta-galactosidase used in the toxicity studies of Coenen *et al.* (2000). The latter, in turn, has amino acid sequence identifies of only 27-35% with the lactases from other microbial species, *Papiliotrema terrestris* (GRN 743), *Bacillus circulans* (GRN 649), *Bifidobacterium bifidum* (GRN 572 and 579) and *Aspergillus oryzae* (GRN 510). This information therefore suggests there is a reasonable expectation of safety for beta-galactosidase produced by *Kluyveromyces lactis* KLA, because it is similar in its amino acid sequence to other beta-galactosidases from *Kluyveromyces lactis* whose safety has been established (*e.g.*, published amino acid sequence, 21 CFR 184.1388, and Coenen *et al.*, 2000).

Microbial beta-galactosidase preparations authorized for use in food in other countries include those produced by *Aspergillus niger*, *Aspergillus oryzae*, *Bacillus circulans* ATCC 31382 and *Kluyveromyces marxianus* in Australia and New Zealand (Standard 1.3.3 processing aids); produced by *Aspergillus niger* var., *Aspergillus oryzae* var., *Kluyveromyces fragilis* (*Kluyveromyces marxianus* var. *marxianus*), *Kluyveromyces lactis* (*Kluyveromyces marxianus* var. *lactis*), *Saccharomyces* sp., and cell-free extracts from *Candida pseudotropicalis* in Canada (*List of Permitted Food Enzymes* at https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html); and those produced by *Aspergillus niger*, *Aspergillus oryzae* and *Kluyveromyces fragilis* in France (Arrêté du 19 Octobre 2006).

In addition to preparations used in food processing, lactase preparations have been sold directly to consumers as tablets, capsules, powders, *etc.* for decades, as a digestive aid for individuals that have difficulty tolerating lactose-containing foods. For example, a search of the published scientific literature revealed studies dating back to the 1980s and 90s of lactase enzyme preparations sold under the names Lactogest, Lactaid, Lactrase, and DairyEase, and Lactase N (Rosado *et al.*, 1984; Moskovitz *et al.*, 1987; Sanders *et al.*, 1992; Lin *et al.*, 1993; Ramirez *et al.*, 1994). Adverse effects reported in some of these studies were limited to gastrointestinal discomfort and headache in a few individuals.

[13] http://www.uniprot.org/uniprot/P00723

Aside from the toxicity studies of beta-galactosidase produced by *Kluyveromyces lactis* LCT described previously (Coenen *et al.*, 2000), the following toxicity studies were identified in the published scientific literature.

A beta-galactosidase enzyme preparation produced by *Penicillium multicolor* was tested in adult and juvenile rats with durations from 35 days up to 6 months. In addition, a 30-day dog study was performed, and reproductive and developmental studies were performed in rats and rabbits. In all animal studies, the NOAEL was the highest dose tested, and it was concluded that the beta-galactosidase preparation can be safely consumed (Flood and Kondo, 2004).

Zou *et al.* (2014) investigated the safety of beta-galactosidase derived from *Aspergillus oryzae* and produced by *Pichia pastoris*. The beta-galactosidase showed no mutagenic activity in an Ames test or a mouse sperm abnormality test at levels of up to 5 mg/plate and 1250 mg/kg body weight, respectively. It also showed no genotoxic activity in a bone marrow cell micronucleus test at levels of up to 1250 mg/kg body weight. During the acute oral toxicity study, none of the rats showed signs of toxicity (dosage was equivalent to beta-galactosidase present in 1500 L milk for a 50-kg human). A 90-day sub-chronic repeated toxicity study via the diet with beta-galactosidase levels up to 1646 mg/kg (1000-fold greater than the mean human exposure) did not show any treatment-related, significant toxicological effects on body weight, food consumption, organ weights, hematological and clinical chemistry, or histopathology compared to the control group. This toxicological evaluation showed no genotoxic, acute, or sub-chronic toxicity for beta-galactosidase under the test conditions used.

The safety of beta-galactosidase produced by *Papiliotrema terrestris* was evaluated by Ke *et al.* (2018). Results for the enzyme concentrate were negative in a bacterial reverse mutation test (Ames test) and a chromosomal aberration test in cultured Chinese hamster lung fibroblast (CHL/IU) cells. In a 13-week oral gavage study in Sprague-Dawley rats, no adverse effects were observed in any of the tested groups, and a No Observed Adverse Effect Level (NOAEL) of 2000 mg/kg bw/day [total organic solids (TOS) 1800 mg/kg bw/day] was established, which was the highest dosage tested. The data support the safety of beta-galactosidase in food production.

Given the long history of use of beta-galactosidase, and based on the fact that none of the safety studies showed any signs of toxicity, it can be concluded that use of beta-galactosidase for the intended purpose is safe. The multiple toxicity studies described in the paragraphs above tested beta-galactosidase from different sources, and all studies concluded that there is no safety concern. The dietary exposure to the food enzyme beta-galactosidase is discussed in section 3.0.

6.5. Allergenicity

Enzymes are proteins with highly specialized catalytic functions. They are produced by all living organisms and are responsible for many essential biochemical reactions in microorganisms, plants, animals, and human beings. Enzymes are essential for all metabolic processes and they have the unique ability to facilitate biochemical reactions without undergoing change themselves. As such, enzymes are protein molecules that act as very efficient catalysts of biochemical reactions. Due to the specific nature of enzymes, only small amounts are required to make desired modifications to the property of a food.

As a protein, enzymes have the potential to cause allergic responses. Although virtually all allergens are proteins, it is noteworthy that only a small percentage of all dietary proteins are food allergens.

Enzymes have a long history of safe use in food. Since new enzymes are generally (based on) existing enzymes, it is very unlikely that a new enzyme would be a food allergen. Moreover, exposure to an enzyme associated with ingestion is typically very low and residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system (Grimble, 1994). To our knowledge, no reports exist on sensitization to enzyme products in the final commercial food after ingestion. Bindslev-Jensen et al. concluded that ingestion of food enzymes in general is not considered to be a concern with regards to food allergy (Bindslev-Jensen et al., 2006).

For the purpose of this dossier, the amino acid sequence of the beta-galactosidase protein produced by the genetically engineered strain of *Kluyveromyces lactis* was compared with the amino acid sequences of known (food) allergens stored in the database AllergenOnline™ (available at http://www.allergenonline.org/, last updated January 18, 2017). AllergenOnline™ allows the search in NCBI, SwissProt, PIR, PRF, PDB and the WHO-IUIS databases using a FASTA algorithm. This comparison did not reveal any relevant matches with known food allergens. Based on these results, it is concluded that the beta-galactosidase protein has no relevant match with known (food) allergens and is not likely to produce an allergenic or sensitization response upon oral consumption (Annex 3).

With regard to allergenicity of the fermentation media, DSM has concluded that the data that it has and the public data and information allow to conclude that there is no published or unpublished data that suggest there is a potential allergen from the fermentation media in the finished enzyme product. To reach that conclusion, DSM relied on the following data:

1. The Enzyme Technical Association in 2004 conducted a survey of its members, and collected information on the possible presence of protein from the fermentation media in the final enzyme product. ETA provided the supporting data and information to FDA in a letter in 2005, and sent an accompanying public statement which is posted on ETA's website (ETA, 2005). The statement concludes that no potentially allergenic protein from the fermentation medium has been found in the finished enzyme. Further, ETA points out that the typical manufacturing process of enzyme preparations includes a step to separate the biomass and fermentation media from the enzyme. This step ensures the enzyme product's purity and stability, and would likely remove most proteins present in the fermentation media.

2. In addition, the Food Allergy Research and Resource Program (FARRP) issued a paper in August of 2013 which concluded that because of the nature of enzymes as catalysts, they are used in very small amounts, and that the fermentation media are consumed during the enzymatic process (FARRP, 2013). It is clear that any de minimis amount of protein present in the fermentation media that survived the fermentation process will not cause a significant public health risk to the consumer. FARRP also underscored the fact that the proteins would likely be removed during the filtration of the enzyme product, as discussed by ETA. Further, FARRP indicated that there is no reliable assay that could be

used to detect the presence of most allergenic proteins in the final enzyme products, as the proteins would likely be degraded into fragments that would not reach levels of quantitation accessible with current commercial ELISA assays. The full August 2013 statement clearly concludes that any protein allergen present in the final enzyme product would not be present at a level that requires labeling or raises a public health concern.

It is therefore concluded that the beta-galactosidase protein is not likely to produce any allergenic or sensitization reactions by oral consumption.

6.6. Safety of the manufacturing process

The manufacture of the beta-galactosidase is performed under current food GMP requirements and, in addition, the HACCP principles are followed (see section 2.5). Moreover, it is indicated that ingredients are used that are acceptable for general use in foods, under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for the production of microbial enzymes.

The beta-galactosidase preparation meets the general and additional quality requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the USP Food Chemicals Codex.

6.7. Substantial equivalence

Several expert groups have discussed the concept of substantial equivalence relative to food safety assessment. Essentially, all these groups conclude that if a food ingredient is substantially equivalent to an existing food ingredient known to be safe, then no further safety considerations other than those for the existing ingredient are necessary.

In addition, FDA appears to have accepted this concept in the determination that several enzyme preparations are safe for use in food. In particular, FDA has considered differences in glycosylation between enzyme proteins. FDA has also stated that enzyme proteins demonstrated to be substantially equivalent to enzymes known to be safely consumed but having differences in specific properties due to chemical modifications, or site-directed mutagenesis, would not raise safety concerns.

There are no agreed-upon criteria by which substantial equivalence is determined. Considering enzymes produced by micro-organisms, the enzyme activity and intended use, the production organism and the process conditions should be taken into account. Beta-galactosidase (EC 3.2.1.23) belongs to the glycosidases (EC 3.2.1). Enzymes from this enzyme class, including beta-galactosidase (lactase), have been notified as GRAS to the FDA: amylases and xylanases, amongst others. For all these enzymes, FDA responded with letters stating they had no questions.

In addition to the safety of the beta-galactosidase enzyme itself, the current production organism is a specific, genetically engineered *Kluyveromyces lactis* strain. *Kluyveromyces lactis* is a common food constituent of fermented dairy products like yogurt, cheese and buttermilk. In addition, *Kluyveromyces lactis* has been used for years to produce enzymes to be used in the food industry. Olempska-Beer et al. (2006) from the FDA summarized the safety of microorganisms, including *Kluyveromyces lactis,* used as hosts for enzyme-encoding genes.

Consequently, the *Kluyveromyces lactis* strain used to produce beta-galactosidase is as safe as the production strains that have produced other enzymes considered as GRAS. Accordingly, it can be concluded that the resulting enzyme product from the production strain is as safe as other enzymes produced by strains from this species.

Since the production strain and production process are as safe as those used to produce other enzymes considered as GRAS, and the native beta-galactosidase (lactase) itself is GRAS according to 21 CFR 184.1388, it can be concluded that the beta-galactosidase from *Kluyveromyces lactis* strain KLA is GRAS.

6.8. Summary of the basis for a GRAS conclusion

DSM has determined that its beta-galactosidase (lactase) enzyme preparation from a genetically engineered strain of *Kluyveromyces lactis*, KLA, is a GRAS substance for use in the manufacture of dairy products including infant and follow-on formula. DSM's GRAS conclusion is based on the totality of available information.

The enzyme preparation is intended to be added to milk and other dairy products, to reduce lactose. The enzyme is either heat denatured and inactivated during the pasteurization/sterilization step, or irreversibly denatured at low pH in the environment of fermented milk products. The use of beta-galactosidase can thus be regarded as a processing aid because it has no function in the finished food.

Beta-galactosidase is an enzyme present in nature, including in humans. The host organism used for production of DSM's enzyme, *Kluyveromyces lactis*, is a well characterized, non-pathogenic organism that has a long history of safe use in the manufacture of food products, and particularly enzymes. This species has been used for many years as a source of beta-galactosidase (lactase) used for conversion of lactose to galactose and glucose in milk and milk products. The beta-galactosidase (lactase) enzyme preparation from *Kluyveromyces lactis* was affirmed by FDA as GRAS (21 CFR 184.1388) in 1984.

Thorough literature searches have been done on the pathogenicity of the *Kluyveromyces lactis*, which provided very little evidence for potential concern.

Beta-galactosidase enzyme preparations from various other microbial sources also have a long history of safe use in food processing, as noted by Pariza and Foster (1983) and Pariza and Johnson (2001), further supported by the authorized uses in the U.S., including applications in both food and dietary supplements.

DSM's beta-galactosidase produced by *Kluyveromyces lactis* KLA is considered substantially equivalent in its composition (including amino acid sequence), function, and conditions of use to other beta-galactosidase (lactase) enzyme preparations whose safety has been established, including the lactase enzyme preparation[14] derived from *Kluyveromyces lactis* that was affirmed

[14] FDA describes the GRAS-affirmed lactase enzyme preparation from *K. lactis* as "a soluble enzyme preparation. It is composed of the cellular fraction of *K. lactis* that contains lactase enzyme activity, residual amounts of processing aids used to separate this cellular fraction from the yeast cells, and substances added to this cellular fraction as stabilizers or diluents. The lactase enzyme preparation

as GRAS (49 FR at 47384, Dec. 4, 1984), and the enzyme produced by *Kluyveromyces lactis* (LCT) that was the subject of toxicity studies reported by Coenen *et al.* (2000).

In assessing the effect of the developed production organism on the safety of the enzyme preparation, DSM considered that the genetic modifications made to *Kluyveromyces lactis* KLA have been well characterized and do not lead to production of harmful or toxic substances. Genomic sequencing of the final production strain and comparison to the *Kluyveromyces lactis* reference genome confirmed the integration of seven copies of the variant beta-galactosidase at the targeted integration sites, no ectopic integration, deletion of the arylsulfatase, invertase and native beta-galactosidase genes, and absence of any selection markers and vector sequences.

A comparison of the amino acid sequence to known food allergens did not reveal any relevant matches.

With respect to consumer exposures, DSM notes that beta-galactosidase produced by *Kluyveromyces lactis* strain KLA represents another type of lactase that will have to compete in essentially the same markets and foods as lactases currently in use. As such, as a replacement for other lactases currently in use for dairy processing, DSM's enzyme would not be expected to significantly impact background consumer exposures to lactase.

Although DSM estimated consumer exposures of 3.7 mg TOS/kg bw/day from use of the enzyme to process milk and other dairy products for the general population, and 9.6 mg TOS/kg bw/day from its use to process milk for infant formula, these estimates are based on conservative assumptions (see section 3.1.3) and represent highly exaggerated values.

While not direct evidence of the safety of beta-galactosidase from *Kluyveromyces lactis* KLA, DSM considered that the NOAEL value of 1540 mg TOS/kg bw/day from the 28-day rat oral toxicity study of beta-galactosidase produced by *Kluyveromyces lactis* LCT (Coenen *et al.*, 2000) might serve as a useful reference for context. The two enzymes are produced by organisms developed by DSM that have common ancestry, have a comparable amino acid sequence, and are produced under similar fermentation conditions, with comparable impurity profiles. If beta-galactosidase produced by *Kluyveromyces lactis* KLA were to yield comparable results in a 28-day rat oral toxicity study, DSM's intake estimates for the general population and infants would be 416 and 160 times lower, respectively, than the NOAEL of 1540 mg TOS/kg bw/day.

Combined, the elements described in this dossier support DSM's conclusion that beta-galactosidase from a genetically engineered strain of *Kluyveromyces lactis* is a GRAS substance for use in the manufacturing of dairy products, including infant and follow-on formula.

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contains the enzyme β-galactoside galactohydrase which catalyzes the hydrolysis of the disaccharide lactose to themonosaccharides glucose and galactose."

7.0 SUPPORTING DATA AND INFORMATION

7.1. List of References

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8.0 LIST OF ANNEXES

Annex 1 – Method of analysis for beta-galactosidase activity

Annex 2 – Flow diagram of the beta-galactosidase manufacturing process

Annex 3 –Allergenicity screening of beta-galactosidase from *Kluyveromyces lactis* strain KLA

Annex 1

Annex 1: Method of analysis of beta-galactosidase activity

METHOD OF ANALYSIS
Spectrophotometric determination of neutral lactase activity using lactose as substrate, absolute manual method

PRODUCT	VALIDATED METHOD
Maxilact SMART in-process samples Maxilact SMART final products	YES

Primary author	Approvers	Status & Dates	Content owner
Stor, Mark	Laumen, Frank, Vink, Collin	**Status: Effective** **Effective: 13/Nov/2017** **Next Review Date: 13/Aug/2020**	Vink, Collin
Reviewers: Laumen, Frank, Peri, Kameshwara			

1 SAFETY, HEALTH AND ENVIRONMENT

Information about hazard symbols, description of H-phrases, risk class information, handling conditions and additional PPE's for working with the chemicals mentioned in this method of analysis can be found in the table in annex 1.

Combination of chemicals and reagents
The combination of chemicals and reagents used in this method of analysis has been considered not to cause safety risks.

Chemical spills
See DBC-SLD-P-10052 "SHE Book Service Lab Delft".
The spills that may occur in the analysis performed according to this method do not require additional measures.

(Set-up of) equipment
The equipment used in this method of analysis, in combination with the chemicals and reagents used, has been considered not to cause safety risks.

Waste
See DBC-SLD-P-10052 "SHE Book Service Lab Delft".
Waste generated in the analysis performed according to this method does not require additional measures.

2 PRINCIPLE

2.1 Application

This method is applicable for the determination of (Kluyveromyces lactis) neutral lactase (ß-galactosidase) activity in fermentation samples (lysed or unlysed K. lactis broth), downstream processing samples and formulated finished products of the Maxilact SMART® range of (intermediate) products.

2.2 Description of the method

Neutral lactase is incubated with lactose at pH 6.45 at 37°C and releases glucose and galactose from this substrate. The lactase reaction is stopped by addition of 0.5N HCl. The released glucose is a measure for enzymatic activity and is determined using the Glucose Hexokinase FS kit. The released glucose is

converted into gluconate-6-P in two steps, during which NADH is formed. The resulting absorbance increase at a wavelength of 340 nm is a measure for glucose released. Lactase activity is calculated using a glucose calibration line.

2.3 Unit definition

Lactase activity is expressed in Lactose Hydrolysis Units per gram or per milliliter. One LHU is defined as the amount of enzyme that liberates 0.81 µmole glucose per minute using lactose substrate under the conditions of the test (pH = 6.45 and T = 37°C).

2.4 Measuring range

The measuring range of this method is from 0.08 to 0.49 LHU per mL.

2.5 Summary of the validation report

Relative Standard Deviation	Maxilact SMART	Maxilact SMART CAPUA Final intermediate	Broth Supernatant	CcuF Stab
Average Activity (LHU/g)	7640	11117	1491	24875
overall	1.88%	1.79%	2.56%	2.86%
within days	1.35%	1.63%	0.71%	1.34%
between days	1.56%	0.90%	2.92%	3.01%
intermediate $_{single}$ (n=1)	2.06%	1.86%	3.01%	3.29%
intermediate $_{duplicate}$ (n=2)	**1.83%**	**1.46%**	**2.96%**	**3.15%**
intermediate $_{triplicate}$ (n=3)	1.74%	1.30%	2.95%	3.11%

Method is robust for small variations (5%) in buffer components, substrate concentration, Monoreagent concentration, and pH (±0.05).

3 APPARATUS AND CONDITIONS

3.1 Apparatus

- Balance, readable to within 0.0001 g : Mettler AE 240
- Balance, readable to within 0.01 g : Sartorius model 2004 MP
- Water bath maintained at 37.0°C ± 0.20°C : Grant W28
- Diluter, provided with 0.5 and 5.0 mL cylinders : Hamilton, Microlabs 600 series
- Multipette : Eppendorf
- pH Meter : Metrhom 827 pH lab
- Vortex mixer : Genie-2, Scientific Industries
- Pipettors, adjusted at 0.1 mL, 0.2 mL, 5 mL and 10 mL : Eppendorf
- Magnetic stirrer : Variomag
- Disposable glass tubes (16 X 100 mm) : VWR 212-0016
- Spectrophotometer adjusted at 340 nm provided with 10 mm UV cuvettes : Shimadzu UV-1800

Or equivalent equipment.

METHOD OF ANALYSIS
Spectrophotometric determination of neutral lactase activity using lactose as substrate, absolute manual method

3.2 Conditions

Set the water bath at 37.0 ± 0.20°C.
Set the spectrophotometer at 340 nm and zero against air after appropriate warm-up time.

4 MATERIALS

4.1 Chemicals

- MES Free acid	: Merck 475893
- Tris(hydroxymethyl)aminomethane	: Merck 1.08382
- Glucose Hexokinase FS kit	: Diasys Diagnostics 1 2511
- Lactose Monohydrate	: Merck 1.07660
- 0.5N Hydrochloric Acid	: Merck 1.09058
- Di-sodium ethylenediaminetetraacetate 2aq (Titriplex III, EDTA)	: Merck 1.08418
- 1-octanol	: Merck 8.20931
- Triton X-100	: Merck 1.12298
- 1,4-dithiothreitol	: Merck 1.11474
- Magnesium chloride hexahydrate	: Merck 1.05886
- Potassium chloride	: Merck 1.04936

Or equivalent quality.

4.2 References, standards and controls

Standard:
Use a glucose standard with an officially assigned content, distributed by the DFS ES Standards Coordinator. Store the amounts for daily single use under the conditions mentioned on the certificate of analysis.

Control:
Only use the neutral lactase control preparation with an officially assigned activity, distributed by the DFS ES Standards Coordinator. Store the amounts for daily single use under the conditions mentioned on the certificate of analysis.

4.3 Reagents

- Water:

Ultra-High Quality (UHQ) water, resistance >18.2 mega Ohm/cm and TOC <500 µg/L.

- Magnesium chloride stock solution:
Weigh 5.59 g magnesium chloride hexahydrate and dissolve in approximately 200 mL water in a 250 mL volumetric flask. Make up to volume with water and mix. This solution may be kept for 3 months at room temperature.

- EDTA stock solution:
Weigh 0.24 g di-sodium ethylenediaminetetraacetate 2aq and dissolve in approximately 200 mL water in a 250 mL volumetric flask. Make up to volume with water and mix. This solution may be kept for 1 year at room temperature.

-Buffer solution (pH 6.45)

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Weigh 9.76 g of MES, 6.06 g of TRIS and 7.45 g Potassium Chloride and dissolve in 800 mL water in a 1 L measuring cylinder. Add 10 mL magnesium chloride stock solution and 10 mL EDTA stock solution. Mix well and adjust the pH to 6.45 (±0.05) using HCl. Make up to volume with water and mix. Never use NaOH to correct pH. This buffer can be kept for 3 months in the refrigerator.

-Dilution buffer:
To 250 mL of buffer solution pH 6.45, add 0.38 g 1,4-dithiothreitol and 0.5 g of Triton X-100 and dissolve by mixing. This buffer can be kept for 1 month in the refrigerator.

-Lactose Substrate:
Weigh 3.75 g lactose monohydrate and dissolve in approximately 40 mL buffer solution pH 6.45 in a 50 mL volumetric flask. Make up to volume with buffer solution pH6.45 and mix.

- Monoreagent (glucose hexokinase):
Mix 4 parts of R1 with 1 part of R2 from the glucose hexokinase kit (e.g. 40 mL R1 and 10 mL R2) in a beaker with a final volume of 50 mL (this amount is sufficient for 10 total reagent tubes (5 samples/5 sample blanks) and glucose calibration curve (5 points)). Make just prior to use and keep in the dark (cover in aluminum foil).

-Lysis buffer:
Weigh 8.0 g MES, 6.06 g TRIS and 7.45 g Potassium Chloride and dissolve in 800 mL water in a 1 L measuring cylinder. Add 10 mL magnesium chloride stock solution and 10 mL EDTA stock solution. Mix well and adjust the pH to 7.4 (±0.05) using HCl. Make up to volume with water and mix. Subsequently dissolve 2 g of Triton X-100 in 1 L of this buffer. This buffer can be kept for 3 months in the refrigerator.

5 PROCEDURE

5.1 Preparation

Not applicable.

5.2 Pretreatment reference

Not applicable.

5.3 Pretreatment standard

Weigh approximately 1 g of glucose standard (accurately to within 0.1 mg) and dissolve in approximately 30 mL of buffer solution pH 6.45 (without Triton/DTT) in a 50 mL volumetric flask, make up to volume with the same and mix. Prepare a glucose calibration line by diluting the glucose standard solution in dilution buffer (with Triton/DTT) according to the following table.

Table 1: Dilution table for preparation of the glucose calibration line

			Dilution 1		Dilution 2		
	Dilutions	Glucose [µmol/assay]	glucose standard (µL)	Dilution buffer (µL)	glucose standard (µL)	Dilution buffer (µL)	Total dilution factor
S0	0	0	0	2000			0
S1	15	0.074	200	2800			750
S2	25	0.044	100	2400			1250

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S3	50	0.022	100	4900			2500
S4	100	0.011	200	1800	200	1800	5000

5.4 Pretreatment control

Before use, allow the control preparation to attain room temperature. Weigh an amount equivalent to 7500 LHU in duplicate in 50 mL volumetric flasks and dissolve in approximately 40 ml of buffer solution pH 6.45 (without Triton/DTT). Make up to volume and mix. Using the diluter, dilute 0.200 mL of each of the solutions with 4.800 mL dilution buffer (with Triton/DTT) and mix. Further, dilute 0.250 mL of each of the solutions with 4.750 mL dilution buffer and mix. Store all diluted solutions in a bath with melting ice until starting the analysis.

5.5 Pretreatment samples

5.5.1 Samples to be weighed

Allow the samples to attain room temperature before use. Weigh at least 1 g sample (note the weight with an accuracy of 1 mg) in a 50 mL volumetric flask. Dissolve in buffer solution pH 6.45 (without Triton/DTT), make up to volume with the same buffer and mix. Dilute these solutions with dilution buffer (with Triton/DTT) to obtain dilutions containing approx. 0.28 LHU/mL.

5.5.2 Samples to be lysed

1. After thorough homogenization of the broth sample, weigh approximately 5 g of broth (record the weight with an accuracy of 1 mg) into a 100 mL volumetric flask and suspend in 90 ml lysis buffer using a dispenser.
2. Add 100 µL of octanol (insert pipette tip into the liquid to ensure proper mixing with broth) while stirring (500 rpm).
3. Start the timer.
4. When octanol is added to all the samples, stir the overall sample set vigorously for 30 sec (~1000 rpm).
5. Leave (in a fume hood) for a minimum of 16 hours and a maximum of 24 hours at room temperature while stirring (500 rpm).
6. Make up to volume precisely (removing the magnet) with lysis buffer, mix, and dilute the sample solutions with dilution buffer (with Triton/DTT) to obtain final sample dilutions containing approx. 0.28 LHU/mL.

5.6 Preparation measurement

5.6.1 Preparation:

To all standard, sample and blank tubes, add 800 µL of substrate with the aid of a multipette. To all standards and blank tubes, also add 300 µL of 0.5N HCl solution and mix.

5.6.2 Sample tubes:

At time t = 0 minutes, put the racks with sample tubes into the water bath at 37°C. Also put the beaker containing the Monoreagent (protected from light) in the water bath.
At time t = 5.0 minutes, in the order of the series and with regular time intervals, pipette 200 µL of sample solution in the substrate and mix.

At time t = 15.0 minutes, in the same order and with the same regular time intervals terminate the incubation by adding 300 µl of 0.5N HCl solution with a multi-pipette and mix.

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Reset the timer and at t = 0 minutes, add 3.00 ml of preheated Monoreagent to each of the reagent tubes. At t = 15 minutes, remove the tubes from the water bath and equilibrate the samples to room temperature for a few minutes.

5.6.3 Sample Blanks and glucose standard solutions:
At t = 0 minutes, pipette 200 µL of standard and blank solutions to each tube and mix. Transfer the rack with blank and standard tubes to the water bath (37°C).
At t = 5.0 minutes add 3.00 ml of preheated Monoreagent to each of the tubes. At t = 20 minutes, remove the tubes from the water bath and equilibrate the samples to room temperature for a few minutes.

5.7 Measurement

Transfer the contents of each tube to a UV-cuvette and determine the absorbance at 340 nm within 30 minutes.

6 CALCULATION

6.1 Calculation of the slope of the glucose calibration line

Calculate the amount of glucose that is present in the assay for the different calibration points (in µmol):

[glucose in assay] = (W x P)/ Df / MW x 1000 (x 1000 / 1000) * Vs

Where

W	=	weight of glucose [g]
P	=	purity of glucose [%]
Df	=	total dilution factor [mL]
MW	=	molecular weight of glucose (180.16) [g/mol]
1000	=	conversion factor from g/mL to mg/mL
1000	=	conversion factor from mol/L to mmol/L or µmol/mL
1000	=	conversion factor from µmol/mL to µmol/µL
Vs	=	volume of sample in assay (200) [µL]

Calculate the corrected A340nm for the calibration samples by subtracting the S0 A340 nm value from the S1 to S4 values. Prepare a calibration line by plotting the calculated amount of glucose versus the corrected absorbance at 340 nm.
This calibration curve must be fitted applying linear regression using Y = aX + b in which Y = corrected A_{340} and X = amount of glucose (µmol). The slope 'a' of this line is used to determine the activity in unknown samples of interest.

6.2 Activity calculation

Activity is calculated as follows:

$[(A_{340nm\ sample} - A_{340nm\ blank}) / a] \times (1000 / Vs) \times 0.81 \times Df / W / t = [LHU/g]$

or

$[(A_{340nm\ sample} - A_{340nm\ blank}) / a] \times (1000 / Vs) \times 0.81 \times Df / t = [LHU/mL]$

where:

$A_{340nm\ sample}$ = sample response in test LHU S

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$A_{340nm\ blank}$	=	blank sample response in test LHU B
a	=	the slope of the calibration line (see 6.1) [abs/µmol]
1000 / Vs	=	conversion factor to mL sample (1000/200) [mL^{-1}]
0.81	=	factor from unit definition
Df	=	Total dilution factor sample [mL]
W	=	Sample weight [g]
t	=	incubation time (10) [min]

7 ASSESSMENT

7.1 Requirements

- Measured activity of a sample solution must be within the measuring range.
- The level of each control value must fit in the range: $C_{assigned} \pm 3 \times SD_{overall}$ ($C_{assigned}$ = Assigned control value; $SD_{overall}$ = overall standard deviation of the average control value calculated from past series).
- The relative (absolute) difference in level between (duplicate) control values within a daily series is not allowed to exceed a value of 2.77 x RSDwithin day. (Relative absolute difference in control values = $(|control\ value\ 1 - control\ value\ 2| / Average\ control\ value) \times 100\ \%$; $RSD_{within\ day}$ = relative overall standard deviation "within a day" calculated from past series using control values e.g. as determined in validation of the method).
- The relative (absolute) difference in level between (duplicate) sample values is not allowed to exceed a value of 2.77 x $RSD_{within\ day}$. (Relative absolute difference in sample values = $(|sample\ value\ 1 - sample\ value\ 2| / Average\ sample\ value) \times 100\ \%$; $RSD_{within\ day}$ = relative overall standard deviation "within a day" calculated from past sample series with a comparable type of matrix e.g. as determined in validation of the method).
- A calibration line point exceeding 5% deviation (after fitting) should be discarded and should be considered non-valid (= outlier). Refitting of the calibration curve is required. Sometimes the deviating point is not the actual outlier, but is appearing as an outlier due to a different point in the line being deviating. By careful examination of the complete line, determine the real outlier.
- The fitted calibration line should consist of at least 80% of the calibration curve points and have an $r^2 \geq 0.999$.

The results of the control sample must be expressed as percentage of the assigned value.
The results of the control samples must be imported into the control charts available for this method of analysis. All results have to be evaluated.

7.2 Actions

- Repeat a sample analysis with an adjusted dilution when results (of the diluted sample) are out of the measuring range.
- Repeat the series whenever the glucose calibration curve does not comply with the requirements.
- Repeat a sample series analysis (completely) when control values do not comply with the requirements.
- Repeat analysis of a sample exceeding the "difference of duplicate" requirement.

7.3 Authorization

After a training period by a for this method authorized technician, the technician will be authorized for this method when she/he succeeds in performing the test single-handedly whereby the selected samples meet the criteria as stated in section 7.1.

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8 REFERENCES

- K. Peri. Development of Lactose Hydrolysis Unit (LHU) activity assay for the analysis of lactase activity in Maxilact SMART. November 2017

9 REMARKS

Traces of iodine and hypochlorite in the ppm range are known to interfere with the lactase activity determination. When it is suspected that glassware is contaminated with hypochlorite, it is allowed to use dilution solution directly in the weighing flask (instead of buffer solution pH6.45).

Sample weights of 1 g in a 50 mL flask are mentioned. For QC analyses, it is allowed to scale up to 2 g in 100 mL if wanted. Scaling down is not allowed.

10 ANNEXES

Annex 1: Chemical abstract table
Annex 2: Example of a glucose calibration line.

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Annex 1: Chemical abstract table

Name	Hazard symbol(s)	Description of H phrases	Risk Class	Handling as such	Handling dilution or solution	Additional PPE's
MES Free acid	NA	NA	NA	Lab Table	Lab Table	NA
Tris	NA	NA	NA	Lab Table	Lab Table	NA
Glucose Hexokinase FS kit	NA	NA	NA	Lab Table	Lab Table	NA
Lactose Monohydrate	NA	NA	NA	Lab Table	Lab Table	NA
Hydrochloric Acid 0.5N		H290 May be corrosive to metals	NA	Lab Table	Lab Table	NA
Di-sodium ethylenediaminetetra acetate 2aq		H332 Harmful if inhaled. H373 May cause damage to organs (Respiratory Tract) through prolonged or repeated exposure if inhaled.	2	Powder cabinet Tox lab	Lab Table	Nitrile gloves
1-octanol		H319 Causes serious eye irritation. H412 Harmful to aquatic life with long lasting effects.	NA	Fume Hood	Fume Hood	Nitrile Gloves
1,4-dithiothreitol		H302 Harmful if swallowed. H315 Causes skin irritation. H319 Causes serious eye irritation	NA	Fume Hood	Lab Table	Nitrile Gloves
Triton X-100		H302 Harmful if swallowed. H315 Causes skin irritation. H318 Causes serious eye damage. H410 Very toxic to aquatic life with long lasting effects	3	Lab Table	Lab Table	Nitrile Gloves
Magnesium chloride hexahydrate	NA	NA	NA	Lab Table	Lab Table	NA
Potassium chloride	NA	NA	NA	Lab Table	Lab Table	NA
Maxilact		H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled	NA	Granulates/ liquids: Lab table Powder: Powder cabinet Tox lab	Lab table	Nitrile gloves

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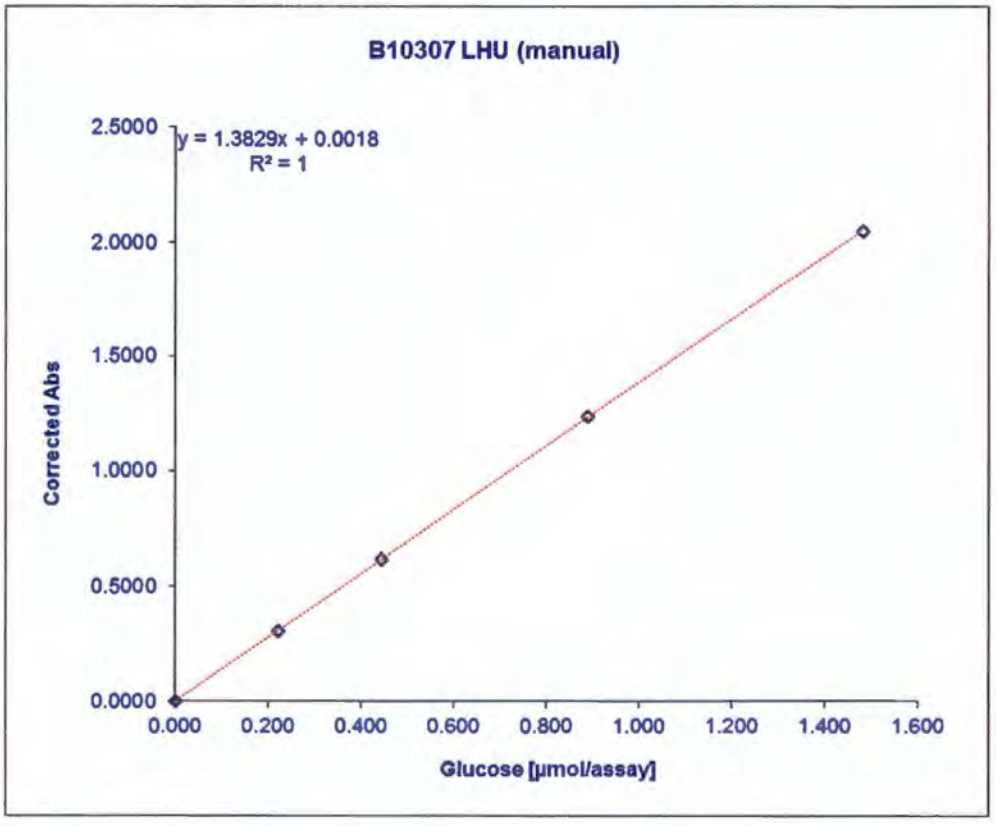
Annex 2: Example of a glucose calibration line.



11 TRAINING

Is training of this document needed?
☐ no

☒ yes → select the type of training:

☐ Training Compass (default); see DBC-ALL-P-00046 mention roles beneath:
.....
.....

☒ ~~Classroom~~ / On the job training

Frequency of training (default is 3 years): See par. 7.3

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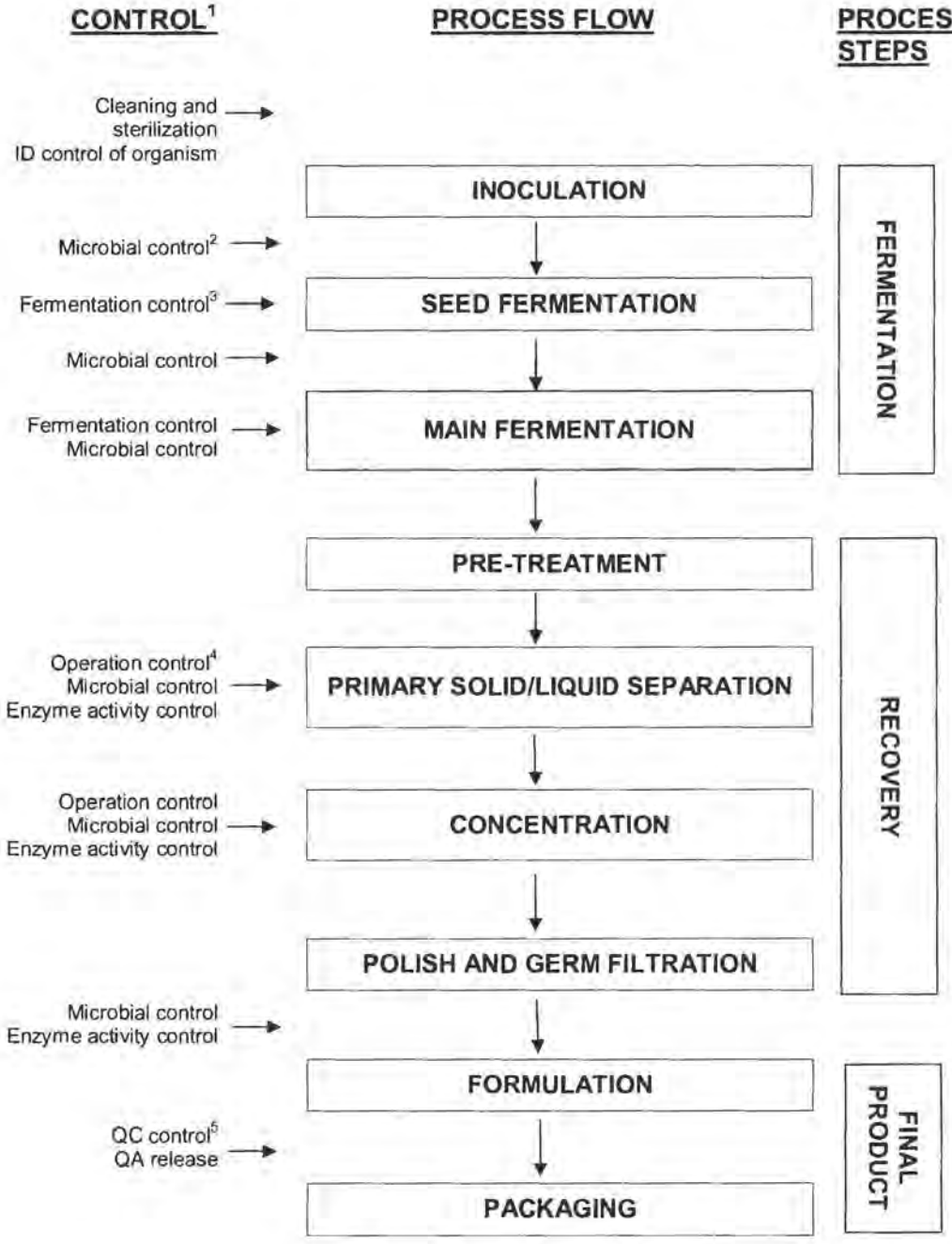
HISTORY

Version	Description of the modification
1	First version

Annex 2

ANNEX 2 Enzyme production flow-chart

Production Process of Food Enzymes from Fermentation



[1] The controls shown on the flow chart may vary depending on the production set-up. Controls are conducted at various steps throughout the production process as relevant.

[2] Microbial control: Absence of significant microbial contamination is analyzed by microscope or plate counts

[3] During fermentation parameters like e.g. pH, temperature, oxygen, CO_2, sterile air overflow are monitored / controlled.

[4] Operation control in downstream processes cover monitoring and control of parameters like e.g. pH, temperature

[5] Final QC control will check that product does live up to specifications like e.g. enzyme activity as well as chemical and microbial specification.

Annex 3

ANNEX 3: Bioinformatics testing for putative allergenicity

Applicant M. van den Dungen

Project# RD.0707.03
Scientist Hilly Menke
Date 26-06-2017

Used allergenicity database:
Database: AllergenOnLine
Last updated: January 18, 2017
Link: http://www.allergenonline.org/

Info of tested protein

Enzyme name	beta-galactosidase
Donor organism	*Kluyveromyces lactis*
Production organism	*Kluyveromyces lactis* strain (KLA)

Results

1. Blast against appropriate database
   As a control a blastP search is performed to verify the sequence and its origin.

Table 1: Blast results

Query sequence	Best hit	Donor organism	Database	% identity	Description
beta-galactosidase	KLLA0B14883g	*Kluyveromyces lactis*	BioIT platform	99 %	B-galactosidase

2. Check for signal sequence
   The signal sequence is removed upon secretion and is supposed to be degraded. Therefore it is assumed that the signal sequence is not present in the product. The signal prediction is performed with SignalPv2.0.

Table 2: Info signal sequence

Query sequence	Signal	length	Signal sequence
beta-galactosidase	No		

3. Full fasta alignment
   Full fasta alignment is used to identify allergens that are highly similar to the query sequence. Matches with E -values > 1 are not likely to be related in evolution or structure while matches with E -values > 10^{-7} are not likely to share immunologic or allergic cross-reactivity.

Table 3: Overview of full fasta alignment

Query sequence	Allergen	Source link	Sequence length	E-score
beta-galactosidase	No hit			

4. FASTA alignments for an 80 amino acids sliding window
   No matches with more than 35% Identity were found between the query sequence and allergens in the Allergen Online database using a window of 80 amino acids and a suitable gap penalty.

Table 4: Hit info alignments for 80 aa sliding window

Query sequence	Allergen	Source link	% age identity	Allergen host	Allergen type
beta-galactosidase	No hit				

5. Exact match for 8 Contiguous amino acids
   No exact matches for 8 contiguous amino acids have been found between the query sequence and all ALLERGEN ONLINE allergens.

Table 5: Hit info exact match for 8 aa

Query sequence	Allergen	Source link	Allergen host	Allergen type
beta-galactosidase	No hit			

6. Available information of detected allergens (hits)

Not applicable

Conclusion

The potential allergenicity of beta-galactosidase from genetically engendered *Kluyveromyces lactis* strain KLA was evaluated by comparing the amino acid sequence of the enzyme with known (food) allergens. For the comparison we made use of the database AllergenOnline.

No stretches of 80 amino acids could be identified with an identity of more than 35% to protein sequences in the AllergenOnline database.

No identical stretches of 8 amino acids or more could be detected in the enzyme sequence as compared to the protein sequences in the AllergenOnline database.