June 7, 2018

APPENDIX A: Technical information

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1 Identity

1.1 Lactase

The systematic name of the principle enzyme activity is β-D-galactoside galactohydrolase. Other names used are: β-galactosidase, Exo-(1->4)-beta-D-galactanase, beta-galactosidase, lactase (ambiguous), beta-lactosidase, maxilact, hydrolact, beta-D-lactosidase, S 2107, lactozym, trilactase, beta-D-galactanase, oryzatym, sumiklat, Milky Whey, CB108 Lactase.

The CB108 Lactase is derive from *Bacillus subtilis* which is genetically modified to overexpress the lactase gene from *Bifidobacterium bifidum.*

- EC number: 3.2.1.23 (Appendix A1)
- CAS number: 9031-11-2 (Appendix A2)

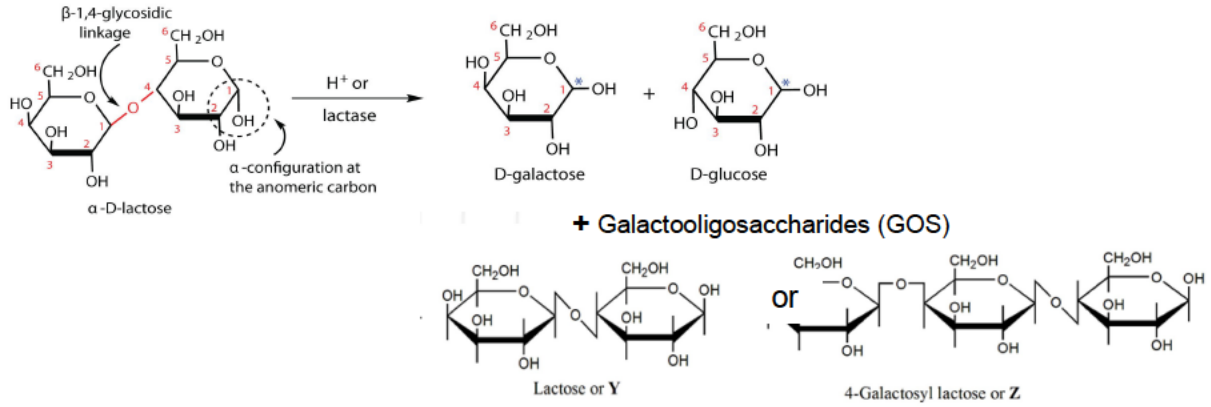
1.2 Other enzymes

Downstream processing concentrates and purifies the enzyme product. The resulting enzyme preparation will not be totally pure and traces of other enzyme activities (e.g. protease) might be found but their level will be very low.

2 Chemical and physical properties

2.1 Substrate specificity

The CB108 Lactase enzyme hydrolyzes the terminal non-reducing β-D-galactose residues in β-D-galactosides. It can use galactosides like lactose as a substrate which is hydrolyzed into galactose and glucose. Galacto-oligosaccharides (GOS) are made up of Galactose and Glucose units. See enzymatic reaction below:



Y (galactosyl acceptor)	Z (product)
Glucose	Lactose
Galactose	Gal-Gal
Lactose	Galactosyl-lactose β-(1,X); (X= 2, 3, 4 or 6)
GOS	$[Gal]_n$-Glc (1=n=6)

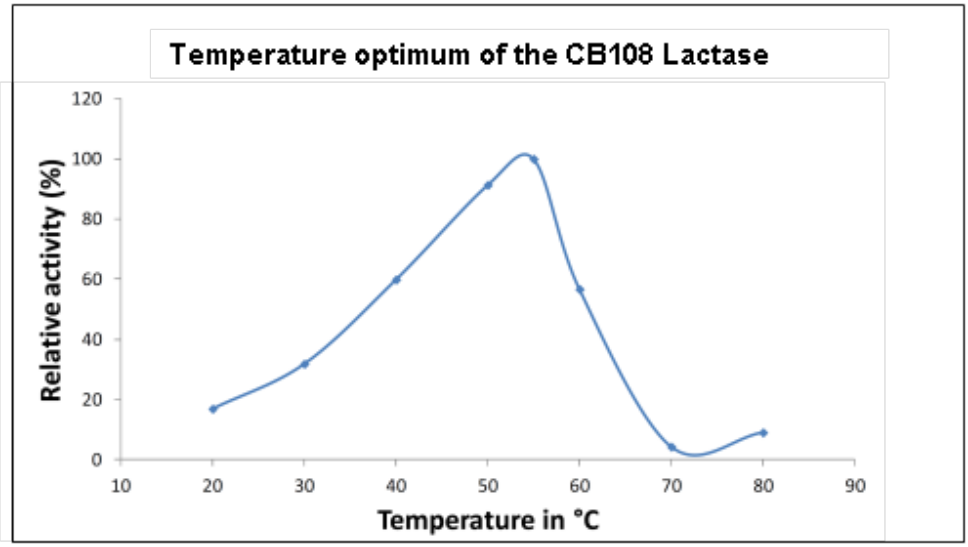
Souza *et al.*, 2015

2.2 Activity

The activity of CB108 Lactase is defined in BLU units/g. The principle of this assay method is that lactase hydrolyzes 2-nitrophenyl-β-D-galactopyranoside (ONPG) into 2-nitophenol (ONP) and galactose. The reaction is stopped after fifteen minutes with the sodium carbonate and the liberated ONP is measured in spectrophotometer. The activity of CB108 Lactase is approximately 540-760 BLU/g enzyme. A detailed assay method is present in Appendix A3.

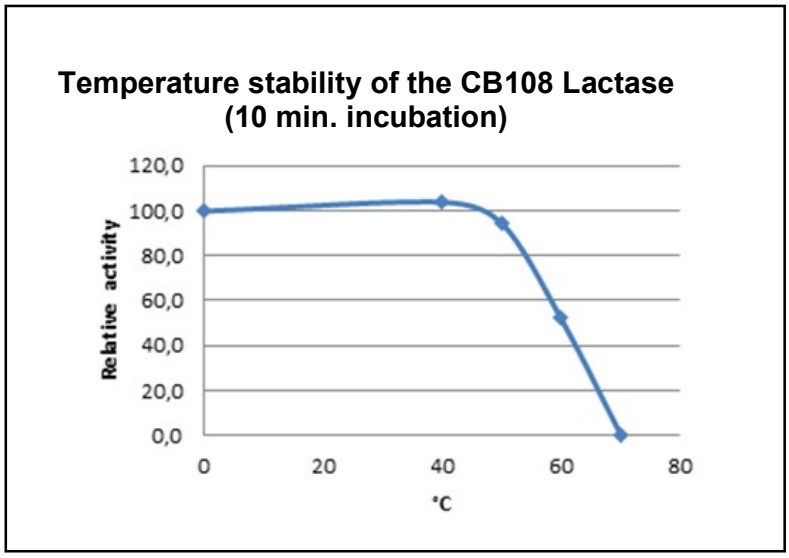
2.3 Temperature optimum

The temperature optimum of the lactase was found by measuring hydrolysis of the colorless substrate 2-nitrophenyl β-D-galactopyranoside (ONPG) into yellow 2-nitrophenol (ONP) and galactose in 100 mM 2-ethanesulfonic (MES) buffer pH 6.4 at varying temperatures. The reaction was stopped after 10 minutes with 10% sodium carbonate and the liberated ONP was measured. Temperature optimum was determined to be about 55°C. At temperatures above 55°C the activity readily decreases and at 70°C less than 10% of the optimum activity is retained.



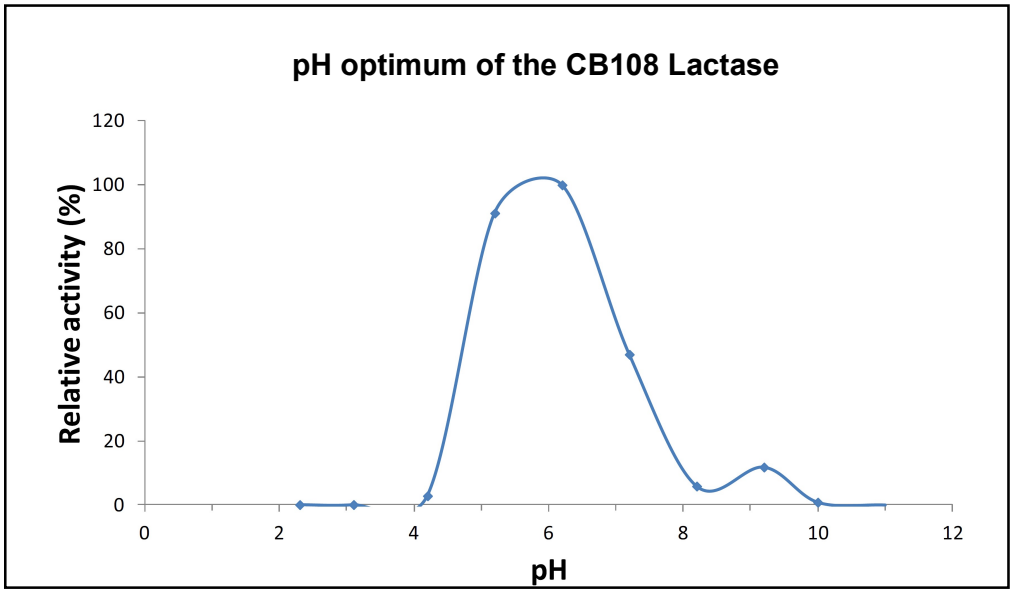
2.4 Thermal stability

Thermal stability of the lactase was measured by determining residual lactase activity after incubation for 10 minutes at temperatures ranging from 0-80°C in 100 mM MES buffer pH 6.4. The enzyme is stable for 10 minutes at temperatures up to 50°C while it is inactivated after 10 minutes' incubation at 70°C, see figure below.



2.5 pH optimum

The pH optimum for CB108 Lactase was determined by measuring hydrolysis of the colorless substrate 2-nitrophenyl β-D-galactopyranoside (ONPG) into yellow 2-nitrophenol (ONP) and galactose at 30°C in 100 mM MCILvane pH 2.3-8.2 and Glycine- NaOH pH 9.2-11. Activity was measured over 10 minutes and the reaction was terminated by addition of 10% sodium carbonate and the samples were measured at 420 nm in an enzyme-linked immunosorbent assay (ELISA) reader.
The optimum pH is 6.2 and the enzyme is active in the range from pH 4 to 8.



2.6 Storage stability

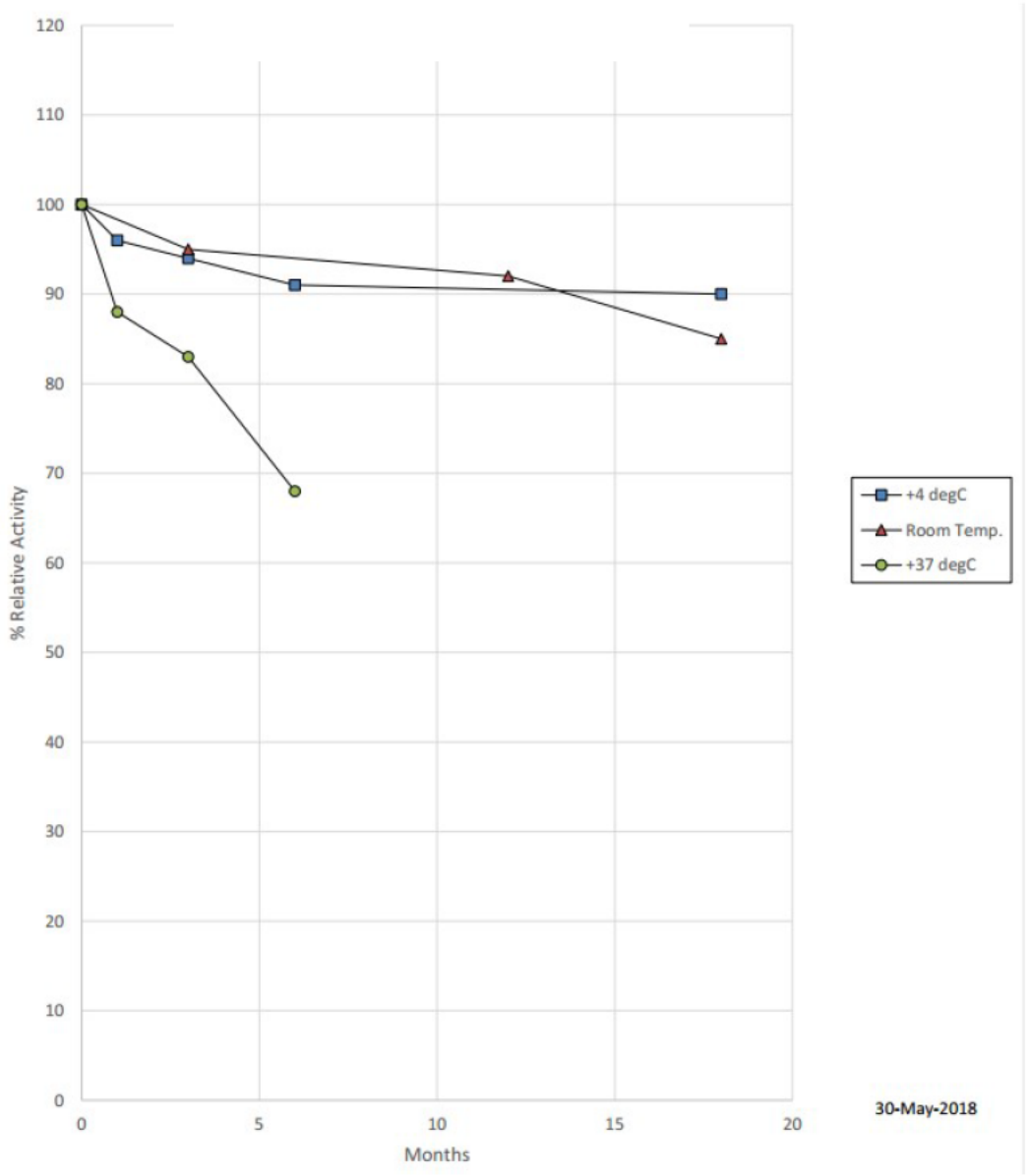


Figure 4. Storage stability of CB108 Lactase

As seen in the figure above, at 4°C, the enzyme is stable for 18 months without significant loss of activity.

3 Efficacy and benefits of the CB108 Lactase enzyme preparation

3.1 Description

As noted above, the function of CB108 Lactase is to hydrolyzes the terminal non-reducing β-D-galactose residues in β-D-galactosides. It can use galactosides like lactose as a substrate which is hydrolyzed into galactose and glucose. Galacto-oligosaccharides (GOS) are made up of Galactose and Glucose units.

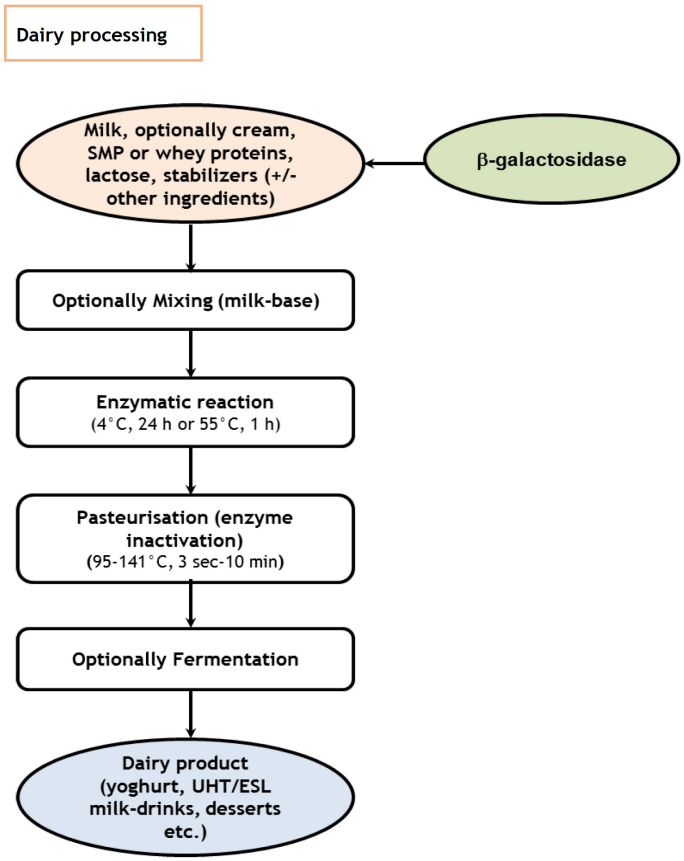
Dairy processing:

In the production of dairy products, the enzyme will convert lactose into GOS and glucose. The use of lactases to deplete lactose from milk is extensive in the dairy industry as a response to the commonly occurring lactose intolerance. However, lactases have not been widely used for GOS production *in situ* in dairy products mainly due to the fact that higher lactose content is needed to generate GOS. Their use in milk whey permeate has been more common than in fresh milk products (Rodriguez-Colinas *et al.*, 2014). Non-digestible fibers added to fresh dairy have primarily been chicory root derived inulin or fructo-oligosaccharides (Charalampopoulos & Rastall, 2012; Meyer *et al.*, 2011). CB108 Lactase will be able to generate GOS *in situ* in raw milk and whey even with low lactose content (5% lactose content in milk), with as benefit to provide 1) a low lactose/lactose free dairy product with reduced caloric content in the final dairy product and 2) enable dairy products to contain prebiotic material.

Below are charts illustrating the anticipated process for using our lactase enzyme preparation in the production of various dairy products, including those with reduced lactose content.

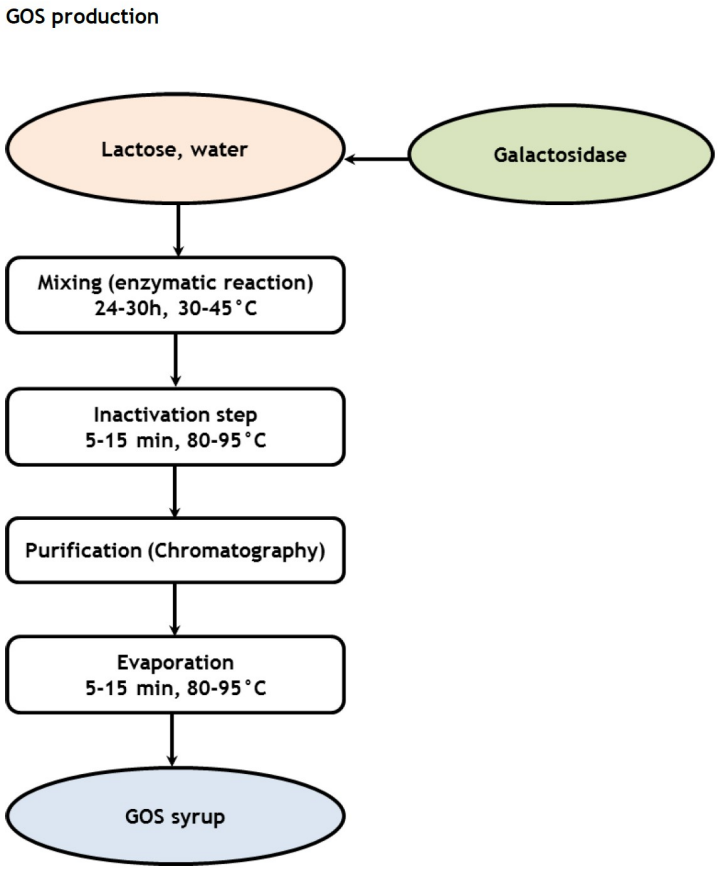
GOS production:

CB108 Lactase will also be used in the production of purified GOS. Because of the configuration of their glycosidic bonds, GOS largely resist hydrolysis by salivary and intestinal digestive enzymes. GOS are classified as prebiotics, defined as non-digestible food ingredients that beneficially affect the host by stimulating the growth and/or activity of beneficial bacteria in the colon. The increased activity of these health-promoting bacteria



results in a number of effects, both directly by the bacteria themselves or indirectly by the organic acids they produce via fermentation. Examples of effects are stimulation of immune functions, absorption of essential nutrients, and syntheses of certain vitamins. GOS is primarily used in infant formula to mimic the effect of the human milk oligosaccharides (HMOs) on babies. These HMOs are known to exert numerous benefits to breast-fed infants. Among others, it has been shown that the incorporation of GOS into infant formula favors the microbiota composition in the infant´s feces and reduces allergenic manifestation and infections during the first years of life (Rodriguez-Colinas *et al.*, 2014). GOS are present in both human milk and bovine milk, but in low concentration especially in human milk. Because CB108 Lactase shows a low degree of hydrolysis and a high degree of transgalactosylation that is not dependent on the initial lactose concentration, the GOS yield in dairy products is directly increased in comparison to other lactases available on the market.

In these applications, the enzyme product will be used in raw milk, whey, or lactose, where, upon heating, the enzyme is not active in the final food, with any remaining inactive protein having no function in the final food.



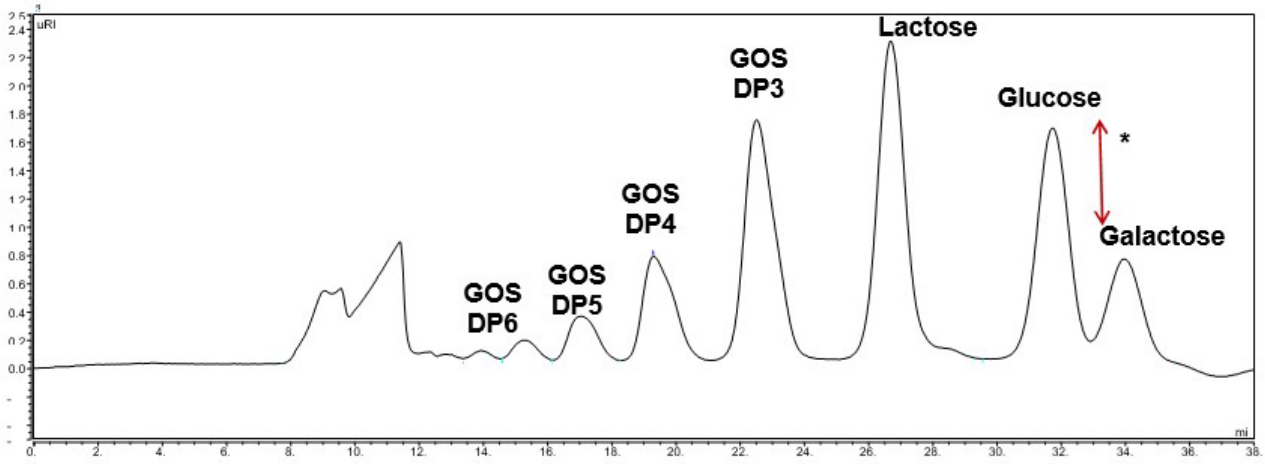
Efficacy examples

Evaluation of dairy processing and GOS production by CB108 Lactase, were tested by *in situ* application in different set-style yogurts. In the current example, the CB108 Lactase was added to 7.5% w/v lactose milk-base to generate galacto-oligosaccharides prior to pasteurization at (10 minutes at 95°C). Yoghurt sugar/oligosaccharide composition was analyzed by HPLC on the day after fermentation.

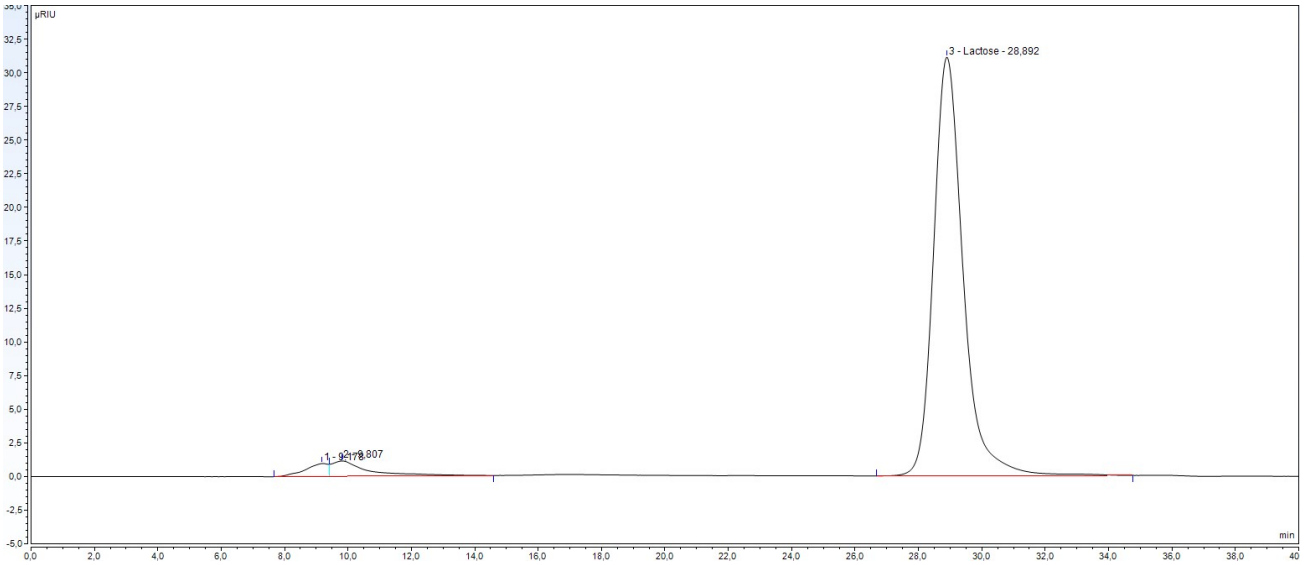
As seen in the HPLC chromatogram below, GOS are detected in the form of DP3 (3-sugar chains) ~ DP6 (6-sugar chains) with the addition of CB108 Lactase, but not detected in the control without lactase. In addition, compared to control, a significant reduction in lactose content been observed in yogurt treated with CB108 Lactase.

HLPC-Chromatogram: *In situ* GOS generation in yogurt

A. treatment with lactose



B. Control without lactase



4 Manufacturing process

The manufacturing process for the production of CB108 LactaseCB108 Lactase will be conducted in a manner similar to other food and feed enzyme production processes. It is conducted in accordance with food good manufacturing practice (GMP) and the resultant product meets the general requirements for enzyme preparations of the Food Chemicals Codex, Sixth Edition (FCC 2008) and the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Join FAO/WHO Expert Committee on Food Additives (JEFCA, 2006).

The manufacturing process is a three-part process consisting of fermentation (growth of organism and production of enzyme), recovery (separation of cell mass from enzyme and concentration/purification of enzyme) and formulation/ drying (preparation of a stable enzyme formulation). The production process follows standard industry practices (see, Enzyme Applications, 1994; Aunstrup et al, 1979; and Aunstrup, 1979).

4.1 Raw materials

The raw materials used in the fermentation and recovery process for the CB108 Lactase enzyme concentrate are standard ingredients used in the enzyme industry. All the raw materials conform to the specifications of the Food Chemical Codex, 6th edition (FCC 2008), except for those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal requirements have been made in line with FCC requirements and acceptability of use for food enzyme production. DuPont IB uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

Full details on raw materials and formulation ingredients used in the production of the enzyme can be found in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

4.2 Fermentation

CB108 Lactase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *B. subtilis* described in Appendix B. The fermentation is an aerobic process and requires continuous addition of air to the fermenter. All equipment is carefully designed, constructed, operated, cleaned and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

The fermentation process consists of three operations: laboratory propagation of the culture, seed fermentation and primary fermentation. These processes, except for the laboratory propagation are carried out in sealed vessels carefully designed to prevent both the release of the production organism and/or the entry of other microorganisms.

A new lyophilized stock culture vial of the *B. subtilis* production organism is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

The fermentation media is sterilised at 121°C for at least 20 minutes. The medium is sampled for microbiological testing prior to inoculation. The fermentation takes place at controlled temperatures.

All stages of the production process are controlled to ensure that the final product conforms to specifications. The culture liquid is sampled at intervals during fermentation for microbiological and enzyme activity tests. Operational parameters such as temperature, pH, air flow, agitation and oxygen content are monitored and controlled to desired values/ranges throughout the fermentation. In addition, at all stages, microbial growth is checked for correct morphological development of the microorganism and for the lack of contamination. Once the fermentation is completed, the fermentation broth is transferred to processing tanks.

4.3 Recovery

The purpose of the recovery process is to separate the biomass, purify, concentrate, and stabilise the desired enzyme, i.e. CB108 Lactase.

Separation of the cell debris from the liquid from the fermentation broth is achieved by either filtration or centrifugation, or a combination of both. Exactly which cell separation technique is used is dependent upon the manufacturing site. The broth may be treated with flocculating agents to maximize separation and is then fed into the filter or the centrifuge. The relatively solids free stream then passes a polishing filter to further clarify the liquid and achieve clear, cell-free filtrate.

The liquid containing the enzyme is concentrated via ultrafiltration, which removes low molecular weight compounds. Diafiltration may follow ultrafiltration to help reach the activity target, remove colour and smaller particles, and carbon treatment may additionally be used to reduce colour. The final recovery step is a polish filtration using either microfiltration membranes, fine filtration aids such as diatomaceous earth or sterile filtration pads.

The ultrafiltered concentrate is then dried and agglomerated using any one of the common drying methods, such as spray drying, fluid bed agglomeration or fluid bed spray drier, or stabilised by e.g. glycerol to produce a liquid product.

A manufacturing flow chart is found in Appendix A6.

4.4 Formulation

The ultrafiltrated concentrate is then formulated and analysed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) and the FCC.

Full details on raw materials and formulation ingredients used in the production of the enzyme can be found in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

5 Specification for identity and purity

5.1 Purity criteria

Appropriate GMP controls and processes are used in the manufacture of CB108 Lactase to ensure that the finished product does not contain any impurities of a hazardous or toxic nature. The specification for impurities and microbial limits for the CB108 Lactase product can be found in Appendix A4. Certificates of Analysis for three lots of product are given in Appendix A5.

The specifications for the CB108 Lactase enzyme preparation meet or exceed the requirements for enzyme preparations as set forth in the Food Chemical Codex, 6th Edition (2008) (Appendix A7) and by the Joint FAO/WHO Expert Committee on Food additives (JECFA 2006) (Appendix A8).

5.2 Allergens

An allergen declaration of the enzyme concentrate can be found in Appendix A9.

6 References

Aunstrup, K. 1979. Production, Isolation, and Economics of Extracellular Enzymes in Applied Biochemistry and Bioengineering, Volume 2, Enzyme Technology, Eds. Wingard, L.B., Katchalski-Katzir, E. and Goldsstein, L. pp. 28-68.

Aunstrup, K., Andersen, O., Falch, E. A., and Nielsen, T. K. 1979. Production of Microbial Enzymes in Microbial Technology, 2nd ed., Volume 1. Eds. Peppler, H.J., and Perlman, D., Chapter 9, pp. 282-309.

Enzyme Applications in Encyclopedia of Chemical Technology, 4th edition. 1994. Kroschwitz, J.I., Volume 9, pp. 567-620.

Food Chemicals Codex (FCC) 6th Edition. 2008. US Pharmacopeia, Rockville, Maryland.

JECFA (Joint FAO/WHO Expert Committee on Food Additives) 2006. General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

Appendix A1 : EC NUMBER

Source: IUBMB / http://www.sbcs.qmul.ac.uk/iubmb/enzyme/EC3/2/1/23.html

Accepted name: β-galactosidase

Reaction: Hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides

Other name(s): lactase; β-lactosidase; maxilact; hydrolact; β-D-lactosidase; S 2107; lactozym; trilactase; β-D-galactanase; oryzatym; sumiklat

Systematic name: β-D-galactoside galactohydrolase

Comments: Some enzymes in this group hydrolyse α-L-arabinosides; some animal enzymes also hydrolyse β-D-fucosides and β-D-glucosides; *cf.* EC 3.2.1.108 lactase.

Links to other databases: BRENDA, EXPASY, GTD, KEGG, Metacyc, PDB, CAS registry number: 9031-11-2

References:

1. Blakely, J.A. and MacKenzie, S.L. Purification and properties of a β-hexosidase from *Sporobolomyces singularis. Can. J. Biochem.* 47 (1969) 1021-1025. [PMID: 5389663]

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3. Kuo, C.H. and Wells, W.W. β-Galactosidase from rat mammary gland. Its purification, properties, and role in the biosynthesis of 6β-*O*-D-galactopyranosyl *myo*-inositol. *J. Biol. Chem.* 253 (1978) 3550-3556. [PMID: 418065]

4. Landman, O.E. Properties and induction of β-galactosidase in *Bacillus megaterium. Biochim. Biophys. Acta* 23 (1957) 558-569.

5. Llanillo, M., Perez, N. and Cabezas, J.A. β-Galactosidase and β-glucosidase activities of the same enzyme from rabbit liver. *Int. J. Biochem.* 8 (1977) 557-564.

6. Monod, J. and Cohn, M. La biosynthèse induite des enzymes (adaptation enzymatique). *Adv. Enzymol. Relat. Subj. Biochem.* 13 (1952) 67-119.

7. Wallenfels, K. and Malhotra, O.P. in Boyer, P.D., Lardy, H. and Myrbäck, K. (Eds.), *The Enzymes*, 2nd edn., vol. 4, Academic Press, New York, 1960, pp. 409-430.

8. Asp, N.G., Dahlqvist, A. and Koldovský, O. Human small-intestinal β-galactosidases. Separation and characterization of one lactase and one hetero β-galactosidase. *Biochem. J.* 114 (1969) 351-359. [PMID: 5822067]

[EC 3.2.1.23 created 1961, modified 1980]

Appendix A2 : CAS NUMBER

Source: http://www.commonchemistry.org/ChemicalDetail.aspx?ref=9031-11-2&title=

CAS Registry Number: 9031-11-2

CA Index Name: Galactosidase, β-

Synonyms:

- 58: PN: WO2006095128 PAGE: 16 claimed sequence
- 64: PN: WO2005111060 TABLE: 2 claimed sequence
- Biolacta
- Biolacta FN 5
- Biolactase L
- E.C. 3.2.1.23
- Fungal lactase 30,000
- galactosidasa, β-
- Galactosidase, β-
- Galaktosidase, β-
- Galantase
- Gamma-lactase A50P
- Hydrolact
- Lactaid
- Lactase
- Lactase F
- Lactase L107P
- Lactase P
- Lactase Y "Amano" L
- Lactase Y-AO
- Lactokanescin G 20x
- Lactoles
- Lactosylceramidase II
- Lactozym 3000 lactase
- Lactozyme
- Lactozyme 3000L
- LX 5000
- Maxilact
- Maxilact 2000
- Maxilact L 2000
- Maxilact LX 5000
- Neutralact
- Oryzatym
- p-Nitrophenyl β-galactosidase
- S 2107
- Sumiklat
- Sumilacto L
- Sumylact GLL
- Sumylact L
- Tilactase
- β-D-Galactopyranosidase
- β-D-Galactoside galactohydrolase
- β-D-Lactosidase
- β-Galactanase
- β-Galactosidase
- β-Lactosidase

- β-L-Galactanase
- γ-Lactase A 50P

Chemical Structure:

Registry Number: 9031-11-2
Formula: Unspecified

No Structure Diagram Available

(disclaimer)

Appendix A3: Activity of the enzyme complex

DANISCO.

Method Number: C986-00
Assay: Lactase, BLU Activity
Effective Date: 16-April-2018

PURPOSE This test method is to be used to determine the activity of Lactase activity in BLU units/g.

NOTE *This assay is suitable for QA/QC monitoring of Lactase products. It is not predictive of the comparative performance of this and other products. Comparative product performance can best be established by actual performance studies under careful experimental control.*

PRINCIPLE The principle of this assay method is that lactase hydrolyzes 2-nitrophenyl-β-D-galactopyranoside (ONPG) into 2-nitophenol (ONP) and galactose. The reaction is stopped after fifteen minutes with the sodium carbonate and the liberated ONP is measured in spectrophotometer.

PROCEDURE

1. Materials and Equipment

- **1.1 Water bath set at 37^0 C**
- **1.2 Vortex**
- **1.3 16 x 100mm glass test tubes**
- **1.4 Timer**
- **1.5 Volumetric flasks, graduate cylinders, beakers**
- **1.6 Magnetic stir bars and stirring/hot plate**
- **1.7 pH meter**
- **1.8 Analytical balance**
- **1.9 Thermometers**
- **1.10 Positive displacement pipets and tips (Ranin Inc.)**
- **1.11 Variable pipetting device (1ml) with disposable tips**
- **1.12 Disposable 1.5 mL visible cuvettes**
- **1.13 Spectrophotometer, capable of reading 420nm**
- **1.14 0.2µm Nalgene filter (1L)**
- **1.15 Calculator**
- **1.16 Aluminum Foil**

DANISCO

Method Number: C986-00
Assay: Lactase, BLU Activity
Effective Date: 16-April-2018

2. Reagents: Use the following reagents or equivalent

2.1 MES hydrate, Sigma Cat # M8250
2.2 Sodium Hydroxide, 10M
2.3 2-nitrophenyl-β-D-galactopyranoside (ONPG), Sigma #N1127
2.4 Sodium Carbonate, monohydrate (Na_2CO_3), Sigma #S4132)
2.5 Deionized Water

3. Reagent Preparation

3.1 0.1M MES buffer (pH 6.4)

Dissolve 19.52 g MES hydrate into 900 ml of de-ionized water. The solution is adjusted to pH 6.4 using 10M sodium hydroxide (NaOH), and brought to volume of 1000 ml. If precipitation appears, filter with 0.2 µm membrane.

3.2 10% Sodium Carbonate Stop Solution

Dissolve 20.0 g of sodium carbonate (Na_2CO_3) into 200 ml of de-ionized water.

3.3 3.7mg/ml ONPG Solution

Dissolve 370 mg of 2-nitrophenol-β-D-galactopyronoside (ONPG) into 100 ml MES buffer. Wrap in foil and store in refrigerator (4°C), for up to 7 days.

DANISCO.

Method Number: C986-00
Assay: Lactase, BLU Activity
Effective Date: 16-April-2018

4. Enzyme Standard Preparation

4.1 Choose a lot of lactase final product as a standard. Set this equal to the BLU value reported on the lot's Certificate of Analysis. Using this material, make a 3 point standard curve using a sample of known concentration in BLU/gram. Dilute the standard accordingly, using MES Buffer so that its net absorbance falls within the linear range of the assay after the subtraction of the reagent blank. The linear range of this assay is 0.50 and 0.90 ΔA. Standard curve and sample concentrations between 0.8 – 1.4 BLU/ml typically fall within the linear range of this assay.

5. Liquid Sample Preparation

5.1 Dilute each sample (w/v) in MES Buffer such that the assay reaction falls between 0.50 and 0.90 ΔA at 540nm.

5.2 Store diluted enzyme solutions on ice. For best results, diluted enzyme solutions should be assayed within 4 hours.

6. Assay Procedure

6.1 Prepare 2 16x100mm glass test tubes for a buffer blank and each sample.

6.2 Equilibrate 0.90 ml of ONPG Substrate at 37°C in glass test tubes for approximately 5 minutes.

6.3 Add 0.10 ml of buffer or enzyme sample dilution at timed intervals. Mix and incubate for exactly 15 minutes.

6.4 Using the same timed intervals stop the reaction by the addition of 1.0ml of the 10% sodium carbonate solution and mix.

6.5 Read the absorbance at 420nm against a de-ionized water blank.

DANISCO

Method Number: C986-00
Assay: Lactase, BLU Activity
Effective Date: 16-April-2018

7. Calculations

7.1 To determine the net ΔAbsorbance, subtract the average buffer blank from the absorbance reading of all standards and samples.

7.2 Prepare a standard curve using linear regression where net absorbance is on the y-axis and concentration (BLU/ml) on the x-axis.

7.3 The correlation coefficient must be ≥0.998.

7.4 Determine the concentration of each sample from linear regression.

7.5 BLU Lactase /g sample =

value from curve (BLU/ml) * sample dilution factor (total volume(ml)/sample weight(g))

Appendix A4: Specification of the commercial product

Property	Reference Method	Specification
ENZYME ACTIVITIES		
Lactase	DuPont Method (via Konelab Activity Assay, See Appendix A3)	Varies with product
MICROBIOLOGICAL ANALYSIS		
Total Viable Count	ISO 4833 - "Microbiology -General guidance for the enumeration of micro-organisms - colony count technique at 30°C" and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 10,000 CFU/mL
E. coli	ISO 7251 - Microbiology - "General Guidance for Enumeration of Presumptive *Escherichia coli* - Most Probable Number Technique" and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Negative/25mL
Total Coliforms	ISO 4832 - "General guidance for the enumeration of coliforms - colony count technique" and the FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 30 CFU/mL
Salmonella	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition; 1991 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Negative/25mL
Production strain	DuPont Method (See "SOP Detection of production organisms, Appendix 16 and results Appendix 12).	Negative by test

Property	Reference Method	Specification
Antibacterial Activity	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p317-318; Appendix A	Negative by test
OTHER ASSAYS		
Heavy Metals as Pb	AAS/ICP-AES method in JECFA, combined compendium of Food Additive Specifications, volume 4, Rome 2006	Less than 30 mg/kg
Arsenic	AAS/ICP-AES method in JECFA, combined compendium of Food Additive Specifications, volume 4, Rome 2006	Less than 3 mg/kg
Cadmium	FAO Food and Nutrition Paper No. 5, GUIDE TO SPECIFICATION, General notices, General analytical techniques, Identification tests, Test solutions, and other reference materials, 1983, 2nd revision	Less than 0.5 mg/kg
Mercury	AAS/ICP-AES method in JECFA, combined compendium of Food Additive Specifications, volume 4, Rome 2006	Less than 0.5 mg/kg
Lead	AAS/ICP-AES method in JECFA, combined compendium of Food Additive Specifications, volume 4, Rome 2006	Less than 5 mg/kg

Appendix A5: Certificates of analyses

DANISCO.

DuPont Industrial Biosciences

1700 Lexington Avenue
Rochester, New York 14606

CERTIFICATE OF ANALYSIS

PRODUCT: **Lactase 14L UFC**

BATCH: **20161029/30**

ASSAY	UNIT	FOUND
ENZYME ACTIVITIES		
Lactase	BLU/g	2792
PHYSICAL PROPERTIES		
pH		5.0
Specific Gravity		1.05
Appearance		Brown liquid
Total Organic Solids	%	16.6
MICROBIOLOGICAL ANALYSIS		
Total Viable Count	CFU/ml	<1
Total Coliforms	CFU/ml	<1
E. coli	/25ml	Negative
Salmonella	/25ml	Negative
Staphylococcus aureus	CFU/ml	Negative
Anaerobic Sulfite Reducing Bacteria	CFU/ml	<1
Production Strain	/ml	Negative
Antibacterial Activity	/ml	Negative
OTHER ASSAYS		
Mycotoxins		
Total Aflatoxin	µg/kg	<5
T-2 toxin	µg/kg	<25
Zearalenone	µg/kg	<50
Ochratoxin	µg/kg	<2
Sterigmatocystin	µg/kg	<250
Fumonisin	µg/kg	<50
Heavy Metals, as Pb	mg/kg	<30
Arsenic	mg/kg	<3
Cadmium	mg/kg	<0.5
Mercury	mg/kg	<0.5
Lead	mg/kg	<5

11-Jan-2017
Date

Manager, Quality Assurance

This certificate of analysis was electronically generated and therefore has not been signed.

QA Form 061-13
26-Mar-2008

DuPont Industrial Biosciences

1700 Lexington Avenue
Rochester, New York 14606

CERTIFICATE OF ANALYSIS

PRODUCT: **Lactase 14L UFC**

BATCH: **20161147/48**

ASSAY	UNIT	FOUND
ENZYME ACTIVITIES		
Lactase	BLU/g	2235
PHYSICAL PROPERTIES		
pH		5.0
Specific Gravity		1.06
Appearance		Brown liquid
Total Organic Solids	%	18.6
MICROBIOLOGICAL ANALYSIS		
Total Viable Count	CFU/ml	<1
Total Coliforms	CFU/ml	<1
E. coli	/25ml	Negative
Salmonella	/25ml	Negative
Staphylococcus aureus	CFU/ml	Negative
Anaerobic Sulfite Reducing Bacteria	CFU/ml	<1
Production Strain	/ml	Negative
Antibacterial Activity	/ml	Negative
OTHER ASSAYS		
Mycotoxins		
Total Aflatoxin	µg/kg	<5
T-2 toxin	µg/kg	<25
Zearalenone	µg/kg	<50
Ochratoxin	µg/kg	<2
Sterigmatocystin	µg/kg	<250
Fumonisin	µg/kg	<50
Heavy Metals, as Pb	mg/kg	<30
Arsenic	mg/kg	<3
Cadmium	mg/kg	<0.5
Mercury	mg/kg	<0.5
Lead	mg/kg	<5

11-Jan-2017
Date

Manager, Quality Assurance

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QA Form 061-13
26-Mar-2008

DuPont Industrial Biosciences

1700 Lexington Avenue
Rochester, New York 14606

CERTIFICATE OF ANALYSIS

PRODUCT: **Lactase 14L UFC**

BATCH: **20161145/46**

ASSAY	UNIT	FOUND
ENZYME ACTIVITIES		
Lactase	BLU/g	2502
PHYSICAL PROPERTIES		
pH		5.0
Specific Gravity		1.06
Appearance		Brown liquid
Total Organic Solids	%	17.9
MICROBIOLOGICAL ANALYSIS		
Total Viable Count	CFU/ml	<1
Total Coliforms	CFU/ml	<1
E. coli	/25ml	Negative
Salmonella	/25ml	Negative
Staphylococcus aureus	CFU/ml	Negative
Anaerobic Sulfite Reducing Bacteria	CFU/ml	<1
Production Strain	/ml	Negative
Antibacterial Activity	/ml	Negative
OTHER ASSAYS		
Mycotoxins		
Total Aflatoxin	µg/kg	<5
T-2 toxin	µg/kg	<25
Zearalenone	µg/kg	<50
Ochratoxin	µg/kg	<2
Sterigmatocystin	µg/kg	<250
Fumonisin	µg/kg	<50
Heavy Metals, as Pb	mg/kg	<30
Arsenic	mg/kg	<3
Cadmium	mg/kg	<0.5
Mercury	mg/kg	<0.5
Lead	mg/kg	<5

11-Jan-2017
Date

Manager, Quality Assurance

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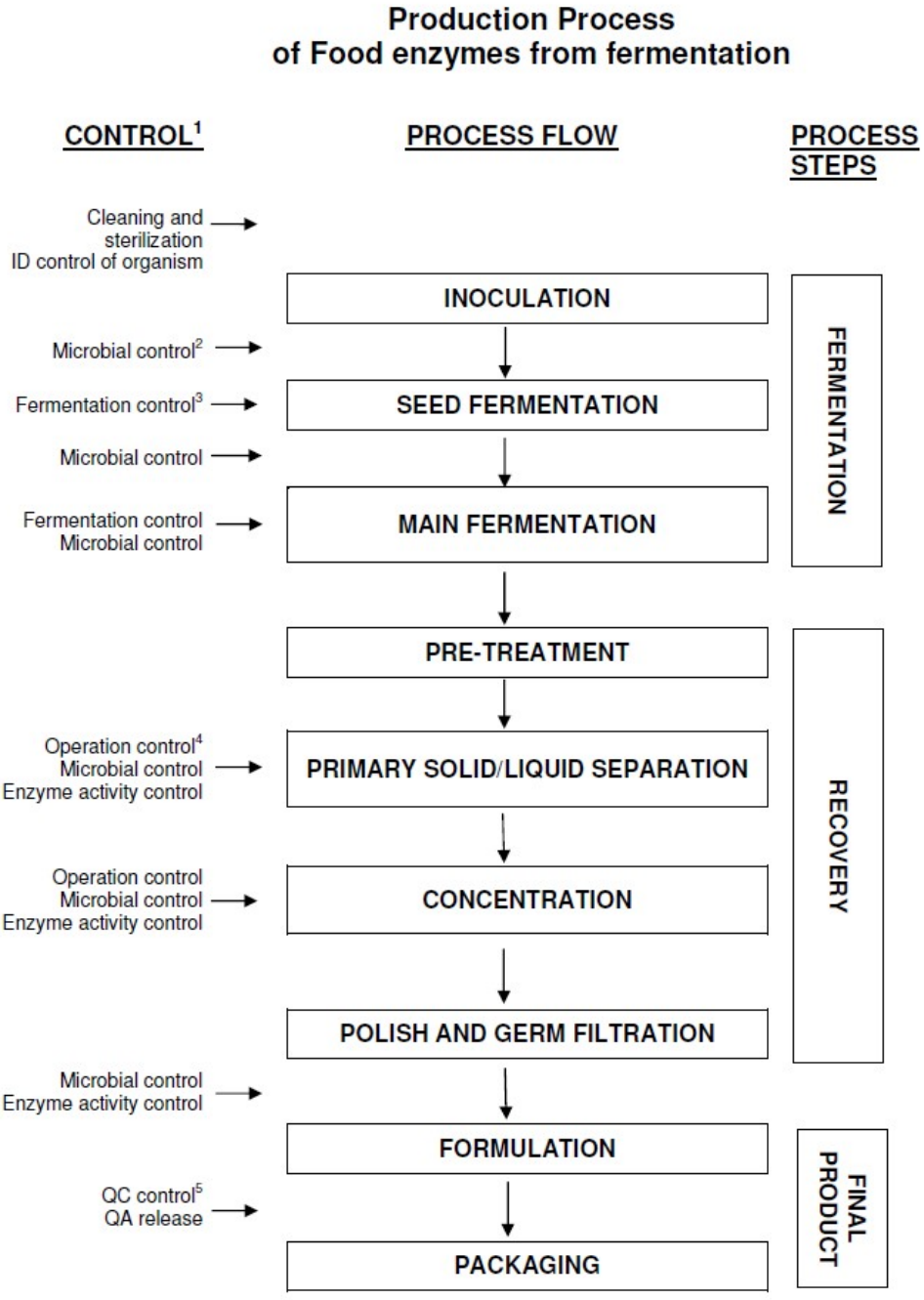
QA Form 061-13
26-Mar-2008

Appendix A6: Production process flow chart



[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 analysed 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.

Appendix A7: Food Chemical Codex, 6th edition

Enzyme Preparations

DESCRIPTION

Enzyme Preparations used in food processing are derived from animal, plant, or microbial sources (see *Classification*, below). They may consist of whole cells, parts of cells, or cell-free extracts of the source used, and they may contain one active component or, more commonly, a mixture of several, as well as food-grade diluents, preservatives, antioxidants, and other substances consistent with good manufacturing practices. The individual preparations usually are named according to the substance to which they are applied, such as *Protease* or *Amylase*. Traditional names such as *Malt, Pepsin*, and *Rennet* also are used, however. The color of the preparations—which may be liquid, semiliquid, or dry—may vary from virtually colorless to dark brown. The active components consist of the biologically active proteins, which are sometimes conjugated with metals, carbohydrates, and/or lipids. Known molecular weights of the active components range from approximately 12,000 to several hundred thousand. The activity of enzyme preparations is measured according to the reaction catalyzed by individual enzymes (see below) and is usually expressed in activity units per unit weight of the preparation. In commercial practice (but not for *Food Chemicals Codex* purposes), the activity of the product is sometimes also given as the quantity of the preparation to be added to a given quantity of food to achieve the desired effect. Additional information relating to the nomenclature and the sources from which the active components are derived is provided under *Enzyme Assays,* Appendix V.

Function Enzyme (see discussion under *Classification*, below)

Packaging and Storage Store in well-closed containers in a cool, dry place.

IDENTIFICATION

Classification

- **ANIMAL-DERIVED PREPARATIONS**

Catalase, Bovine Liver: Produced as partially purified liquid or powdered extracts from bovine liver. Major active principle: *catalase*. Typical application: used in the manufacture of certain cheeses.

Chymotrypsin: Obtained from purified extracts of bovine or porcine pancreatic tissue. Produced as white to tan, amorphous powders soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *chymotrypsin*. Typical application: used in the hydrolysis of protein.

Lipase, Animal: Obtained from the edible forestomach tissue of calves, kids, or lambs; and from animal pancreatic tissue. Produced as purified edible tissue preparations or as aqueous extracts dispersible in water, but insoluble in alcohol. Major active principle: *lipase*. Typical applications: used in the manufacture of cheese and in the modification of lipids.

Lysozyme: Obtained from extracts of purified chicken egg whites. Generally prepared and used in the hydrochloride form as a white powder. Major active principle: *lysozyme*. Typical application: used as an antimicrobial in food processing.

Pancreatin: Obtained from porcine or bovine (ox) pancreatic tissue. Produced as a white to tan, water-soluble powder. Major active principles: (1) α-amylase; (2) protease; and (3)

lipase. Typical applications: used in the preparation of precooked cereals, infant foods, and protein hydrolysates.

Pepsin: Obtained from the glandular layer of hog stomach. Produced as a white to light tan, water-soluble powder; amber paste; or clear, amber to brown, aqueous liquids. Major active principle: *pepsin*. Typical applications: used in the preparation of fishmeal and other protein hydrolysates and in the clotting of milk in the manufacture of cheese (in combination with rennet).

Phospholipase A_2: Obtained from porcine pancreatic tissue. Produced as a white to tan powder or pale to dark yellow liquid. Major active principle: *phospholipase* A_2. Typical application: used in the hydrolysis of lecithins.

Rennet, Bovine: Aqueous extracts made from the fourth stomach of bovines. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: *protease* (pepsin). Typical application: used in the manufacture of cheese. Similar preparations may be made from the fourth stomach of sheep or goats.

Rennet, Calf: Aqueous extracts made from the fourth stomach of calves. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: *protease* (chymosin). Typical application: used in the manufacture of cheese. Similar preparations may be made from the fourth stomach of lambs or kids.

Trypsin: Obtained from purified extracts of porcine or bovine pancreas. Produced as white to tan, amorphous powders soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *trypsin*. Typical applications: used in baking, in the tenderizing of meat, and in the production of protein hydrolysates.

- **PLANT-DERIVED PREPARATIONS**

Amylase: Obtained from extraction of ungerminated barley. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: β-*amylase*. Typical applications: used in the production of alcoholic beverages and sugar syrups.

Bromelain: The purified proteolytic substance derived from the pineapples *Ananas comosus* and *Ananas bracteatus* L. (Fam. Bromeliaceae). Produced as a white to light tan, amorphous powder soluble in water (the solution is usually colorless to light yellow and somewhat opalescent), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *bromelain*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, in the preparation of precooked cereals, in the production of protein hydrolysates, and in baking.

Ficin: The purified proteolytic substance derived from the latex of *Ficus* sp. (Fam. Moraceae), which includes a variety of tropical fig trees. Produced as a white to off-white powder completely soluble in water. (Liquid fig latex concentrates are light to dark brown.) Major active principle: *ficin*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, and in the conditioning of dough in baking.

Malt: The product of the controlled germination of barley. Produced as a clear amber to dark brown liquid preparation or as a white to tan powder. Major active principles: (1) α-*amylase* and (2) β-*amylase*. Typical applications: used in baking, in the manufacture of alcoholic beverages and of syrups.

Papain: The purified proteolytic substance derived from the fruit of the papaya *Carica papaya* L. (Fam. Caricaceae). Produced as a white to light tan, amorphous powder or a liquid soluble in water (the solution is usually colorless or light yellow and somewhat opalescent), but

practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *papain* and (2) *chymopapain*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, in the preparation of precooked cereals, and in the production of protein hydrolysates.

- **MICROBIALLY-DERIVED PREPARATIONS**

α-Acetolactatedecarboxylase: (*Bacillus subtilis* containing a *Bacillus brevis* gene) Produced as a brown liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually a light yellow to brown). Major active principle: *decarboxylase*. Typical application: used in the preparation of beer.

Aminopeptidase, Leucine: (*Aspergillus niger* var., *Aspergillus oryzae* var., and other microbial species) Produced as a light tan to brown powder or as a brown liquid by controlled fermentation using *Aspergillus niger* var., *Aspergillus oryzae* var., or other microbial species. The powder is soluble in water (the solution is usually light yellow to brown). Major active principles: (1) *aminopeptidase*, (2) *protease*, and (3) *carboxypeptidase* activities in varying amounts. Typical applications: used in the preparation of protein hydrolysates and in the development of flavors in processed foods.

Carbohydrase: (*Aspergillus niger* var., including *Aspergillus aculeatus*) Produced as an off-white to tan powder or a tan to dark brown liquid by controlled fermentation using *Aspergillus niger* var. (including *Aspergillus aculeatus*). Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *pectinase* (a mixture of enzymes, including *pectin depolymerase, pectin methyl esterase, pectin lyase*, and *pectate lyase*), (3) *cellulase*, (4) *glucoamylase* (amyloglucosidase), (5) *amylo-1,6-glucosidase*, (6) *hemicellulase* (a mixture of enzymes, including *poly(galacturonate) hydrolase, arabinosidase, mannosidase, mannanase*, and *xylanase*), (7) *lactase*, (8) β-*glucanase*, (9) β-D-*glucosidase*, (10) *pentosanase*, and (11) α-*galactosidase*. Typical applications: used in the preparation of starch syrups and dextrose, alcohol, beer, ale, fruit juices, chocolate syrups, bakery products, liquid coffee, wine, dairy products, cereals, and spice and flavor extracts.

Carbohydrase: (*Aspergillus oryzae* var.) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Aspergillus oryzae* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *glucoamylase* (amyloglucosidase), and (3) *lactase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, ale, bakery products, and dairy products.

Carbohydrase: (*Bacillus acidopullulyticus*) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using *Bacillus acidopullulyticus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *pullulanase*. Typical applications: used in the hydrolysis of amylopectins and other branched polysaccharides.

Carbohydrase: (*Bacillus stearothermophilus*) Produced as an off-white to tan powder or a light yellow to dark brown liquid by controlled fermentation using *Bacillus stearothermophilus*. Soluble in water, but practically insoluble in alcohol, in ether, and in chloroform. Major active principle: α-*amylase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, and bakery products.

Carbohydrase: (*Candida pseudotropicalis*) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Candida pseudotropicalis*. Soluble in

water (the solution is usually light yellow to dark brown) but insoluble in alcohol, in chloroform, and in ether. Major active principle: *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: (*Kluyveromyces marxianus* var. *lactis*) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Kluyveromyces marxianus* var. *lactis*. Soluble in water (the solution is usually light yellow to dark brown), but insoluble in alcohol, in chloroform, and in ether. Major active principle: *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: (*Mortierella vinaceae* var. *raffinoseutilizer*) Produced as an off-white to tan powder or as pellets by controlled fermentation using *Mortierella vinaceae* var. *raffinoseutilizer*. Soluble in water (pellets may be insoluble in water), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: α-*galactosidase*. Typical application: used in the production of sugar from sugar beets.

Carbohydrase: (*Rhizopus niveus*) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using *Rhizopus niveus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase* and (2) *glucoamylase*. Typical application: used in the hydrolysis of starch.

Carbohydrase: (*Rhizopus oryzae* var.) Produced as a powder or a liquid by controlled fermentation using *Rhizopus oryzae* var. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *pectinase*, and (3) *glucoamylase* (amyloglucosidase). Typical applications: used in the preparation of starch syrups and fruit juices, vegetable purees, and juices and in the manufacture of cheese.

Carbohydrase: (*Saccharomyces* species) Produced as a white to tan, amorphous powder by controlled fermentation using a number of species of *Saccharomyces* traditionally used in the manufacture of food. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *invertase* and (2) *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: [(*Trichoderma longibrachiatum* var.) (formerly *reesei*)] Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Trichoderma longibrachiatum* var. Soluble in water (the solution is usually tan to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *cellulase*, (2) β-*glucanase*, (3) β-D-*glucosidase*, (4) *hemicellulase*, and (5) *pentosanase*. Typical applications: used in the preparation of fruit juices, wine, vegetable oils, beer, and baked goods.

Carbohydrase: (*Bacillus subtilis* containing a *Bacillus megaterium* α-*amylase* gene) Produced as an off-white to brown, amorphous powder or liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: α-*amylase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, and dextrose.

Carbohydrase (*Bacillus subtilis* containing a *Bacillus stearothermophilus* α-amylase gene) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: maltogenic *amylase*. Typical applications: used in the preparation of starch syrups, dextrose, alcohol, beer, and baked goods.

Carbohydrase and Protease, Mixed: (*Bacillus licheniformis* var.) Produced as an off-white to brown, amorphous powder or as a liquid by controlled fermentation using *Bacillus licheniformis* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase* and (2) *protease*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, fishmeal, and protein hydrolysates.

Carbohydrase and Protease, Mixed: (*Bacillus subtilis* var. including *Bacillus amyloliquefaciens*) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Bacillus subtilis* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) β-*glucanase*, (3) *protease*, and (4) *pentosanase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, bakery products, and fishmeal, in the tenderizing of meat, and in the preparation of protein hydrolysates.

Catalase: (*Aspergillus niger* var.) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually tan to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *catalase*. Typical applications: used in the manufacture of cheese, egg products, and soft drinks.

Catalase: (*Micrococcus lysodeikticus*) Produced by controlled fermentation using *Micrococcus lysodeikticus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *catalase*. Typical application: used in the manufacture of cheese, egg products, and soft drinks.

Chymosin: (*Aspergillus niger* var. *awamori, Escherichia coli K-12*, and *Kluyveromyces marxianus*, each microorganism containing a calf *prochymosin* gene) Produced as a white to tan, amorphous powder or as a light yellow to brown liquid by controlled fermentation using the above-named genetically modified microorganisms. The powder is soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *chymosin*. Typical application: used in the manufacture of cheese and in the preparation of milk-based desserts.

Glucose Isomerase: (*Actinoplanes missouriensis, Bacillus coagulans, Streptomyces olivaceus, Streptomyces olivochromogenes, Microbacterium arborescens, Streptomyces rubiginosus* var., or *Streptomyces murinus*) Produced as an off-white to tan, brown, or pink amorphous powder, granules, or liquid by controlled fermentation using any of the above-named organisms. The products may be soluble in water, but practically insoluble in alcohol, in chloroform, and in ether; or if immobilized, may be insoluble in water and partially soluble in alcohol, in chloroform, and in ether. Major active principle: *glucose* (or *xylose*) *isomerase*. Typical applications: used in the manufacture of high-fructose corn syrup and other fructose starch syrups.

Glucose Oxidase: (*Aspergillus niger* var.) Produced as a yellow to brown solution or as a yellow to tan or off-white powder by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually light yellow to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *glucose oxidase* and (2) *catalase*. Typical applications: used in the removal of sugar from liquid eggs and in the deoxygenation of citrus beverages.

Lipase: (*Aspergillus niger* var.) Produced as an off-white to tan, amorphous powder by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually

light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical application: used in the hydrolysis of lipids (e.g., fish oil concentrates and cereal-derived lipids).

Lipase: (*Aspergillus oryzae* var.) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Aspergillus oryzae* var. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids (e.g., fish oil concentrates) and in the manufacture of cheese and cheese flavors.

Lipase: (*Candida rugosa;* formerly *Candida cylindracea*) Produced as an off-white to tan powder by controlled fermentation using *Candida rugosa*. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids, in the manufacture of dairy products and confectionery goods, and in the development of flavor in processed foods.

Lipase: [*Rhizomucor (Mucor) miehei*] Produced as an off-white to tan powder or as a liquid by controlled fermentation using *Rhizomucor miehei*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids, in the manufacture of cheese, and in the removal of haze in fruit juices.

Phytase: (*Aspergillus niger* var.) Produced as an off-white to brown powder or as a tan to dark brown liquid by controlled fermentation using *Aspergillus niger* var. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *3-phytase* and (2) *acid phosphatase*. Typical applications: used in the production of soy protein isolate and in the removal of phytic acid from plant materials.

Protease: (*Aspergillus niger* var.) Produced by controlled fermentation using *Aspergillus niger* var. The purified enzyme occurs as an off-white to tan, amorphous powder. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the production of protein hydrolysates.

Protease: (*Aspergillus oryzae* var.) Produced by controlled fermentation using *Aspergillus oryzae* var. The purified enzyme occurs as an off-white to tan, amorphous powder. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical applications: used in the chillproofing of beer, in the production of bakery products, in the tenderizing of meat, in the production of protein hydrolysates, and in the development of flavor in processed foods.

Rennet, Microbial: (nonpathogenic strain of *Bacillus cereus*) Produced as a white to tan, amorphous powder or a light yellow to dark brown liquid by controlled fermentation using *Bacillus cereus*. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Rennet, Microbial: (*Endothia parasitica*) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using nonpathogenic strains of *Endothia parasitica*. The powder is soluble in water (the solution is usually tan to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Rennet, Microbial: [*Rhizomucor (Mucor)* sp.] Produced as a white to tan, amorphous powder by controlled fermentation using *Rhizomucor miehei*, or *pusillus* var. Lindt. The powder is

soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Transglutaminase: (*Streptoverticillium mobaraense* var.) Produced as an off-white to weak yellow-brown, amorphous powder by controlled fermentation using *Streptoverticillium mobaraense* var. Soluble in water but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *transglutaminase*. Typical applications: used in the processing of meat, poultry, and seafood; production of yogurt, certain cheeses, and frozen desserts; and manufacture of pasta products and noodles, baked goods, meat analogs, ready-to-eat cereals, and other grain-based foods.

- **REACTIONS CATALYZED**

[NOTE: The reactions catalyzed by any given active component are essentially the same, regardless of the source from which that component is derived.]

α-Acetolactatedecarboxylase: Decarboxylation of α-cetolactate to acetoin

Aminopeptidase, Leucine: Hydrolysis of *N*-terminal amino acid, which is preferably leucine, but may be other amino acids, from proteins and oligopeptides, yielding free amino acids and oligopeptides of lower molecular weight

α-Amylase: Endohydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding dextrins and oligo- and monosaccharides

β-**Amylase:** Hydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding maltose and betalimit dextrins

Bromelain: Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, leucine, or glycine), yielding peptides of lower molecular weight

Catalase: $2H_2O_2 \leftrightarrows O_2 + 2H_2O$

Cellulase: Hydrolysis of β-1,4-glucan bonds in such polysaccharides as cellulose, yielding β-dextrins

Chymosin (calf and fermentation derived): Cleaves a single bond in kappa casein

Ficin: Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, leucine, or glycine), yielding peptides of lower molecular weight

α-Galactosidase: Hydrolysis of terminal nonreducing α-D-galactose residues in α-D-galactosides

β-Glucanase: Hydrolysis of β-1,3- and β-1,4-linkages in β-D-glucans, yielding oligosaccharides and glucose

Glucoamylase (amyloglucosidase): Hydrolysis of terminal α-1,4- and α-1,6-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding glucose (dextrose)

Glucose Isomerase (xylose isomerase): Isomerization of glucose to fructose, and xylose to xylulose

Glucose Oxidase: β-D-glucose + $O_2 \leftrightarrows$ D-glucono-δ-lactone + H_2O_2

β-D-**Glucosidase:** Hydrolysis of terminal, nonreducing β-D-glucose residues with the release of β-D-glucose

Hemicellulase: Hydrolysis of β-1,4-glucans, α-L-arabinosides, β-D-mannosides, 1,3-β-D-xylans, and other polysaccharides, yielding polysaccharides of lower molecular weight

Invertase (β-fructofuranosidase): Hydrolysis of sucrose to a mixture of glucose and fructose (invert sugar)

Lactase (β-galactosidase): Hydrolysis of lactose to a mixture of glucose and galactose

Lysozyme: Hydrolysis of cell-wall polysaccharides of various bacterial species leading to the breakdown of the cell wall most often in Gram-positive bacteria

Maltogenic Amylase: Hydrolysis of α-1,4-glucan bonds

Lipase: Hydrolysis of triglycerides of simple fatty acids, yielding mono- and diglycerides, glycerol, and free fatty acids

Pancreatin:

α-Amylase: Hydrolysis of α-1,4-glucan bonds

Protease: Hydrolysis of proteins and polypepticles

Lipase: Hydrolysis of triglycerides of simple fatty acids

Pectinase:

Pectate lyase: Hydrolysis of pectate to oligosaccharides

Pectin depolymerase: Hydrolysis of 1,4 galacturonide bonds

Pectin lyase: Hydrolysis of oligosaccharides formed by pectate lyase

Pectinesterase: Demethylation of pectin

Pepsin: Hydrolysis of polypeptides, including those with bonds adjacent to aromatic or dicarboxylic L-amino acid residues, yielding peptides of lower molecular weight

Phospholipase A_2: Hydrolysis of lecithins and phosphatidylcholine, producing fatty acid anions

Phytase:

3-Phytase: *myo*-Inositol hexakisphosphate + H_2O ⇔ 1,2,4,5,6-pentakisphosphate + orthophosphate

Acid Phosphatase: Orthophosphate monoester + H_2O⇔an alcohol + orthophosphate

Protease (generic): Hydrolysis of polypeptides, yielding peptides of lower molecular weight

Pullulanase: Hydrolysis of 1,6-α-D-glycosidic bonds on amylopectin and glycogen and in α- and β-limit dextrins, yielding linear polysaccharides

Rennet (bovine and calf): Hydrolysis of polypeptides; specificity may be similar to pepsin

Transglutaminase: Binding of proteins

Trypsin: Hydrolysis of polypeptides, amides, and esters at bonds involving the carboxyl groups of L-arginine and L-lysine, yielding peptides of lower molecular weight

ASSAY

- **PROCEDURE**

Analysis: The following procedures, which are included under *Enzyme Assays,* Appendix V, are provided for application as necessary in determining compliance with the declared representations for enzyme activity[1]: Acid Phosphatase Activity, α-Amylase Activity (Nonbacterial); Bacterial α-Amylase Activity (BAU); Catalase Activity; Cellulase Activity; Chymotrypsin Activity; Diastase Activity (Diastatic Power); α-Galactosidase Activity, β-Glucanase Activity; Glucoamylase Activity (Amyloglucosidase Activity); Glucose Isomerase Activity; Glucose Oxidase Activity; β-D-Glucosidase Activity; Hemicellulase Activity; Invertase Activity; Lactase (Neutral) (β-Galactosidase) Activity; Lactase (Acid) (β-Galactosidase) Activity; Lipase Activity; Lipase/Esterase (Forestomach) Activity; Maltogenic Amylase Activity; Milk-Clotting Activity; Pancreatin Activity; Pepsin Activity; Phospholipase Activity; Phytase Activity; Plant Proteolytic Activity; Proteolytic Activity, Bacterial (PC); Proteolytic Activity, Fungal (HUT); Proteolytic Activity, Fungal (SAP); Pullulanase Activity; and Trypsin Activity.

Acceptance criteria: NLT 85.0% and NMT 115.0% of the declared units of enzyme activity

IMPURITIES

- **LEAD,** *Lead Limit Test,* Appendix IIIB

Control: 5 µg Pb (5 mL of *Diluted Standard Lead Solution*)

Acceptance criterion: NMT 5 mg/kg

SPECIFIC TESTS

- **MICROBIAL LIMITS**

[NOTE: Current methods for the following tests may be found in the Food and Drug Administration's Bacteriological Analytical Manual online at www.cfsan.fda.gov/.]

Acceptance criteria:

Coliforms: NMT 30 CFU/g

Salmonella: Negative in 25 g

OTHER REQUIREMENTS

Change to read:

Enzyme preparations are produced in accordance with good manufacturing practices. Regardless of the source of derivation, they should cause no increase in the total microbial count in the treated food over the level accepted for the respective food.

Animal tissues used to produce enzymes must comply with the applicable U.S. meat inspection requirements and must be handled in accordance with good hygienic practices.

Plant material used to produce enzymes or culture media used to grow microorganisms consist of components that leave no residues harmful to health in the finished food under normal conditions of use.

▲Preparations derived from microbial sources shall be obtained using a pure culture fermentation of a non-pathogenic and non-toxigenic strain and are produced by methods and under culture conditions that ensure a controlled fermentation, thus preventing the introduction of microorganisms that could be the source of toxic materials and other undesirable substances.▲ *FCC 6*

The carriers, diluents, and processing aids used to produce the enzyme preparations shall be substances that are acceptable for general use in foods, including water and substances that are insoluble in foods but removed from the foods after processing.

Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants.

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[1] Because of the varied conditions under which pectinases are employed, and because laboratory hydrolysis of a purified pectin substrate does not correlate with results observed with the natural substrates under use conditions, pectinase suppliers and users should develop their own assay procedures that would relate to the specific application under consideration.

Auxiliary Information— Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
Monograph		(FI07) Food Ingredients Expert Committee

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Appendix A8: General specifications for enzyme preparations used in food processing (JECFA)

General Specifications and Considerations for Enzyme Preparations Used in Food Processing

The following general specifications were prepared by the Committee at its sixty-seventh meeting (2006) for publication in FAO JECFA Monographs 3 (2006), superseding the general specifications prepared at the fifty-seventh meeting (*1*) and published in FAO JECFA Monographs 1 (*2*). These specifications were originally prepared by the Committee at its twenty-fifth meeting (*3*) and published in FAO Food and Nutrition Papers No. 19 and No. 31/2 (*4,5*). Subsequent revisions were made by the Committee at its thirty-fifth meeting and published in FAO Food and Nutrition Paper No. 52 (*6*). Additional amendments were made at the fifty-first meeting and published in FAO Food and Nutrition Paper No. 52 Add. 6 *(7*), and at the fifty-third meeting *(8*) and partially published in FAO Food and Nutrition Paper No. 52 Add. 7 *(9)*.

Classification and nomenclature of enzymes

Enzymes are proteins that catalyse chemical reactions. The Enzyme Commission of the International Union of Biochemistry and Molecular Biology (formerly the International Union of Biochemistry) classified enzymes into six main classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (*10*). Based on the type of reaction catalysed, enzymes are assigned to one of these classes and given an Enzyme Commission (EC) number, a systematic name, and a common name. Other names are also provided, if available. Enzymes used in food processing are often referred to by their common or traditional names such as protease, amylase, malt, or rennet. For enzymes derived from microorganisms, the name of the source microorganism is usually specified, for example, "α-amylase from *Bacillus subtilis*." For enzymes derived from microorganisms modified by using recombinant DNA techniques (referred to as recombinant-DNA microorganisms or genetically modified microorganisms), the names of both the enzyme source (donor organism) and the production microorganism are provided, for example, "α-amylase from *Bacillus licheniformis* expressed in *Bacillus subtilis*."

Enzyme preparations

Enzymes are used in food processing as enzyme preparations. An enzyme preparation contains an active enzyme (in some instances a blend of two or more enzymes) and intentionally added formulation ingredients such as diluents, stabilizing agents, and preserving agents. The formulation ingredients may include water, salt, sucrose, sorbitol, dextrin, cellulose, or other suitable compounds. Enzyme preparations may also contain constituents of the source organism (i.e. an animal, plant, or microbial material from which an enzyme was isolated) and compounds derived from the manufacturing process, for example, the residues of the fermentation broth. Depending on the application, an enzyme preparation may be formulated as a liquid, semi-liquid or dried product. The colour of an

enzyme preparation may vary from colourless to dark brown. Some enzymes are immobilized on solid support materials.

Active components

Enzyme preparations usually contain one principal enzyme that catalyses one specific reaction during food processing. For example, α-amylase catalyses the hydrolysis of 1,4-α-D-glucosidic linkages in starch and related polysaccharides. However, some enzyme preparations contain a mixture of enzymes that catalyse two or more different reactions in food. Each principal enzyme present in an enzyme preparation is characterized by its systematic name, common name, and EC number. The activity of each enzyme is measured using an appropriate assay and expressed in defined activity units per weight (or volume) of the preparation.

Source materials

Enzymes used in food processing are derived from animal, plant, and microbial sources. Animal tissues used for the preparation of enzymes should comply with meat inspection requirements and be handled in accordance with good hygienic practice.

Plant material and microorganisms used in the production of enzyme preparations should not leave any residues harmful to health in the processed finished food under normal conditions of use.

Microbial strains used in the production of enzyme preparations may be native strains or mutant strains derived from native strains by the processes of serial culture and selection or mutagenesis and selection or by the application of recombinant DNA technology. Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis (*11–15*). Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

Microbial production strains should be taxonomically and genetically characterized and identified by a strain number or other designation. The strain identity may be included in individual specifications, if appropriate. The strains should be maintained under conditions that ensure the absence of genetic drift and, when used in the production of enzyme preparations, should be subjected to methods and culture conditions that are applied consistently and reproducibly from batch to batch. Such conditions should prevent the introduction of microorganisms that could be the source of toxic and other undesirable substances. Culture media used for the growth of microbial sources should consist of components that leave no residues harmful to health in the processed finished food under normal conditions of use.

Enzyme preparations should be produced in accordance with good food manufacturing practice and cause no increase in the total microbial count in the treated food over the level considered to be acceptable for the respective food.

Substances used in processing and formulation

Substances used in processing and formulation of enzyme preparations should be suitable for their intended uses.

In the case of immobilized enzyme preparations, leakage of active enzymes, support materials, crosslinking agents and/or other substances used in immobilization should be kept within acceptable limits established in the individual specifications.

To distinguish the proportion of the enzyme preparation derived from the source material and manufacturing process from that contributed by intentionally added formulation ingredients, the content of total organic solids (TOS) is calculated as follows:

% TOS = 100 - (A + W + D)

where:
A = % ash, W = % water and D = % diluents and/or other formulation ingredients.

Purity

Lead:
Not more than 5 mg/kg.
Determine using an atomic absorption spectroscopy/inductively coupled atomic-emission spectroscopy (AAS/ICP-AES) technique appropriate to the specified level. The selection of the sample size and the method of sample preparation may be based on the principles described in the *Compendium of Food Additive Specifications*, Volume 4.

Microbiological criteria:
Salmonella species: absent in 25 g of sample
Total coliforms: not more than 30 per gram
Escherichia coli: absent in 25 g of sample
Determine using procedures described in Volume 4.

Antimicrobial activity:
Absent in preparations from microbial sources.

Other considerations

Safety assessment of food enzyme preparations has been addressed in a number of publications and documents. Pariza & Foster (*11*) proposed a decision tree for determining the safety of microbial enzyme preparations. Pariza & Johnson (*16*) subsequently updated this decision tree and included information on enzyme preparations derived from recombinant-DNA microorganisms. The Scientific Committee on Food (*17*) issued guidelines for the presentation of data on food enzymes. The document includes a discussion on enzymes from genetically modified organisms including microorganisms, plants, and animals. Several international organizations, government agencies, and expert groups have also published discussion papers or guidelines that address safety assessment of food and food ingredients derived from recombinant-DNA plants and microorganisms (*18–28*). Certain information in these documents may be applicable to enzyme preparations derived from recombinant sources.

An overall safety assessment of each enzyme preparation intended for use in food processing should be performed. This assessment should include an evaluation of the safety of the production organism, the enzyme component, side activities, the manufacturing process, and the consideration of dietary exposure. Evaluation of the enzyme component should include considerations of its potential to cause an allergic reaction. For enzyme preparations from recombinant-DNA microorganisms, the following should also be considered:

1. The genetic material introduced into and remaining in the production microorganism should be characterized and evaluated for function and safety, including evidence that it does not contain genes encoding known virulence factors, protein toxins, and enzymes involved in the synthesis of mycotoxins or other toxic or undesirable substances.

2. Recombinant-DNA production microorganisms might contain genes encoding proteins that inactivate clinically useful antibiotics. Enzyme preparations derived from such microorganisms should contain neither antibiotic inactivating proteins at concentrations that would interfere with antibiotic treatment nor transformable DNA that could potentially contribute to the spread of antibiotic resistance.

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Appendix A9 : Allergen declaration on the enzyme preparation

The table below indicates the presence (as added component) of the following allergens and products thereof *. **Unless otherwise noted, the following listed allergens and products thereof have been used in the recovery process or in the formulation of an enzyme product:**

YES	NO	Allergens	Description of components
	(X)	Wheat	Glucose (used in fermentation)**
	X	Other cereals containing gluten	
	X	Crustaceans	
	X	Eggs	
	X	Fish	
	X	Peanuts	
	(X)	Soybeans	Soy (used in fermentation)**
	X	Milk (including lactose)	
	X	Nuts	
	X	Celery	
	X	Mustard	
	X	Sulphur dioxide and sulfites >10mg/kg	
	X	Lupine	
	X	Molluscs	

*Local legislation has always to be consulted as allergen labeling requirements may vary from country to country.

**Danisco has determined that fermentation nutrients are outside the scope of US and EU food allergen labeling requirements