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(71) Applicant: **NOVOZYMES A/S** [DK/DK]; Krogshoejvej
36, 2880 Bagsvaerd (DK).

(72) Inventors: **CARSTENSEN, Lone**; Krogshoejvej 36, 2880
Bagsvaerd (DK). **SPODSBERG, Nikolaj**; Krogshoejvej
36, 2880 Bagsvaerd (DK). **GJERMANSEN, Morten**;
Krogshoejvej 36, 2880 Bagsvaerd (DK). **SALOMON, Jes-
per**; Krogshoejvej 36, 2880 Bagsvaerd (DK). **KROGH,
Kristian, Bertel, Roemer, M.**; Krogshoejvej 36, 2880
Bagsvaerd (DK).

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(54) Title: POLYPEPTIDES HAVING ALPHA-GALACTOSIDASE ACTIVITY AND POLYNUCLEOTIDES ENCODING SAME

(57) Abstract: The present invention relates to methods of releasing galactose from legumes using polypeptides having alpha-galactosidase activity. The invention also relates to polypeptides having alpha-galactosidase activity, polynucleotides encoding the polypeptides, nucleic acid constructs, vectors, and host cells comprising the polynucleotides as well as methods of producing the polypeptides. The invention also relates to compositions comprising the polypeptides of the invention and the use of the polypeptides in animal feed.

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POLYPEPTIDES HAVING ALPHA-GALACTOSIDASE ACTIVITY AND POLYNUCLEOTIDES ENCODING SAME

Reference to a Sequence Listing

5 This application contains a Sequence Listing in computer readable form, which is incorporated herein by reference.

Background of the Invention

Field of the Invention

10 The present invention relates to methods of releasing galactose from legumes using polypeptides having alpha-galactosidase activity. The invention also relates to polypeptides having alpha-galactosidase activity, polynucleotides encoding the polypeptides, nucleic acid constructs, vectors, and host cells comprising the polynucleotides as well as methods of producing the polypeptides. The invention also relates to compositions comprising the polypeptides of the invention and the use of the polypeptides in animal feed.

15 Description of the Related Art

 Soybean is a species of legume native to East Asia and is the second biggest animal feed crop globally and the biggest feed protein source. Soy bean can be manufactured (defatted) to produce soybean meal (SBM), and SBM is a significant and cheap source of protein for animal feeds. Other common types of legume are chickpea, lupin, lentil, peanut, beans or peas which
20 can also be processed and used as animal feed.

 Alpha-galactosidase is a glycoside hydrolase enzyme that hydrolyses the terminal alpha-galactosyl moieties from glycolipids and glycoproteins that is present in, e.g. legumes, vegetables, grains, cereals and the like. Alpha-galactosidases are produced by various microorganisms, plants and animals, but monogastric animals are deficient in intestinal alpha-galactosidase
25 production and consequently are incapable of decomposing ingested alpha-galactosidases by themselves. Instead, ingested alpha-galactosidases are decomposed by microorganisms present in the large intestines, where this microbial fermentation may cause flatulence and possibly wet litter.

 Animal feed supplemented with alpha-galactosidases can help the animal digest the food
30 thereby obtaining a higher release of galactose and sucrose which will promote animal growth (performance). However, the alpha-galactoside needs to survive passage through the stomach, i.e. low pH and proteases such as pepsin, before it can get to work in the intestine. Animal feed,

especially for poultry, is normally pelleted before being fed and the enzyme also needs to survive the pelleting conditions. Thus the object of this invention is to provide acid stable alpha-galactosidases which are highly effective at releasing galactose and sucrose from legumes.

Summary of the Invention

5 The present invention relates to an animal feed additive, granules and liquid formulations comprising one or more GH36 polypeptides having alpha-galactosidase activity selected from the group consisting of:

- 10 (a) a polypeptide having at least 80%, e.g., at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the polypeptide of SEQ ID NO: 3;
- (b) a polypeptide encoded by a polynucleotide that hybridizes under high stringency conditions, or very high stringency conditions with
 - 15 (i) the mature polypeptide coding sequence of SEQ ID NO: 1,
 - (ii) the cDNA sequence thereof, or
 - (iii) the full-length complementary strand of (i) or (ii);
- 20 (c) a polypeptide encoded by a polynucleotide having at least 80%, e.g., at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1;
- 25 (d) a variant of the polypeptide selected from the group consisting of SEQ ID NO: 3, wherein the variant has alpha-galactosidase activity and comprises one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in up to 50 positions;
- (e) a polypeptide comprising the polypeptide of (a), (b), (c) or (d) and a N-terminal and/or C-terminal His-tag and/or HQ-tag;
- 30 (f) a polypeptide comprising the polypeptide of (a), (b), (c) or (d) and a N-terminal and/or C-terminal extension of up to 10 amino acids, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids; and
- (g) a fragment of the polypeptide of (a), (b), (c) or (d) having alpha-galactosidase activity and having at least 90% of the length of the mature polypeptide.

35 The invention further relates to isolated polypeptides having alpha-galactosidase activity as defined in the claims; compositions, such as animal feed, comprising the polypeptide of the

invention and uses thereof; methods of releasing galactose from plant based material; methods of improving the performance of an animal; methods for improving the nutritional value of an animal feed; polynucleotides encoding the polypeptides of the present invention; nucleic acid constructs; expression vectors; recombinant host cells comprising the polynucleotides; methods
5 of producing the polypeptides and uses thereof.

Overview of Sequence Listing

SEQ ID NO: 1 is the cDNA sequence of GH36 alpha-galactosidase as isolated from *Penicillium pseudopulvillum*.

SEQ ID NO: 2 is the amino acid sequence as deduced from SEQ ID NO: 2.

10 SEQ ID NO: 3 is the amino acid sequence of the mature GH36 alpha-galactosidase from *Penicillium pseudopulvillum*.

SEQ ID NO: 4 is the corrected amino acid sequence of the alpha-galactosidase as disclosed in WO1994/23022.

Definitions

15 **Allelic variant:** The term "allelic variant" means any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

20 **Alpha-galactosidase:** The term "alpha-galactosidase", also called α -D-galactoside galactohydrolase (E.C. 3.2.1.22), means an enzyme that catalyses the hydrolysis of terminal, non-reducing α -D-galactose residues in α -D-galactosides, such as galactose oligosaccharides, galactomannans and galactolipids. Alpha-galactosidase activity can be determined using 4-nitrophenyl α -D-galactopyranoside (available from Megazyme International, Bray, Co. Wicklow,
25 Ireland) as substrate in 100 mM MES (Sigma) buffer pH 7.0 \pm 0.05 at room temperature. The enzyme is diluted in 2-fold dilutions and then the 4-nitrophenyl α -D-galactopyranoside substrate is dissolved in the solution containing the enzyme. The alpha-galactosidase activity is followed directly in the buffer by measuring the absorbance of released pNP at 405 nm as function of time. A detailed assay can be found in the alpha-galactosidase assay as described herein.

30 In one aspect, the polypeptides of the present invention have at least 40%, such as at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 100% of the alpha-galactosidase activity of the polypeptide of SEQ ID NO: 3.

35 In one aspect, the polypeptide has improved gastric stability compared to control (wherein control is defined as SEQ ID NO: 4). In an embodiment, the gastric stability is measured as the

half-life at 40°C pH3 with 0.1 mg/ml pepsin. In an embodiment, gastric stability is measured as described in example 4 herein. In a further embodiment, the polypeptide has a half-life of at least 90 minutes, such as 2 hours, 3 hours or 4 hours.

In one aspect, the polypeptide has increased alpha-galactosidase activity compared to control (wherein control is defined as SEQ ID NO: 4). In one embodiment, activity is measured using 4-nitrophenyl α -D-galactopyranoside as substrate. In one embodiment, activity is measured using 4-nitrophenyl α -D-galactopyranoside as substrate (1 mg/ml in 100 mM MES buffer pH 7.0 \pm 0.05, prepared immediately before use) as substrate by measuring the absorbance of released pNP in the buffer at 405 nm for 5 minutes as function of time at room temperature (typically 23°C). In an embodiment, activity is measured as described in example 3 herein. In an embodiment, the polypeptide has an activity of at least 100000 (mOD/min)/(mg/ml), such as at least 150000, at least 200000, at least 250000, or at least 300000 (mOD/min)/(mg/ml) using 4-nitrophenyl α -D-galactopyranoside as substrate.

Animal: The term "animal" refers to all animals except humans. Examples of animals are non-ruminants, and ruminants. Ruminant animals include, for example, animals such as sheep, goats, cattle, e.g. beef cattle, cows, and young calves, deer, yank, camel, llama and kangaroo. Non-ruminant animals include mono-gastric animals, e.g. pigs or swine (including, but not limited to, piglets, growing pigs, and sows); poultry such as turkeys, ducks and chicken (including but not limited to broiler chicks, layers); horses (including but not limited to hotbloods, coldbloods and warm bloods), young calves; fish (including but not limited to amberjack, arapaima, barb, bass, bluefish, bocachico, bream, bullhead, cachama, carp, catfish, catla, chanos, char, cichlid, cobia, cod, crappie, dorada, drum, eel, goby, goldfish, gourami, grouper, guapote, halibut, java, labeo, lai, loach, mackerel, milkfish, mojarra, mudfish, mullet, paco, pearlspot, pejerrey, perch, pike, pompano, roach, salmon, sampa, sauger, sea bass, seabream, shiner, sleeper, snakehead, snapper, snook, sole, spinefoot, sturgeon, sunfish, sweetfish, tench, terror, tilapia, trout, tuna, turbot, vendace, walleye and whitefish); and crustaceans (including but not limited to shrimps and prawns).

Animal feed: The term "animal feed" refers to any compound, preparation, or mixture suitable for, or intended for intake by an animal. Animal feed for a mono-gastric animal typically comprises concentrates as well as vitamins, minerals, enzymes, direct fed microbial, amino acids and/or other feed ingredients (such as in a premix) whereas animal feed for ruminants generally comprises forage (including roughage and silage) and may further comprise concentrates as well as vitamins, minerals, enzymes direct fed microbial, amino acid and/or other feed ingredients (such as in a premix).

Body Weight Gain: The term "body weight gain" means an increase in live weight of an animal during a given period of time e.g. the increase in weight from day 1 to day 21.

cDNA: The term "cDNA" means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic or prokaryotic cell. cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

Coding sequence: The term "coding sequence" means a polynucleotide, which directly specifies the amino acid sequence of a polypeptide. The boundaries of the coding sequence are generally determined by an open reading frame, which begins with a start codon such as ATG, GTG, or TTG and ends with a stop codon such as TAA, TAG, or TGA. The coding sequence may be a genomic DNA, cDNA, synthetic DNA, or a combination thereof.

Control sequences: The term "control sequences" means nucleic acid sequences necessary for expression of a polynucleotide encoding a mature polypeptide of the present invention. Each control sequence may be native (*i.e.*, from the same gene) or foreign (*i.e.*, from a different gene) to the polynucleotide encoding the polypeptide or native or foreign to each other. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the polynucleotide encoding a polypeptide.

Expression: The term "expression" includes any step involved in the production of a polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

Expression vector: The term "expression vector" means a linear or circular DNA molecule that comprises a polynucleotide encoding a polypeptide and is operably linked to control sequences that provide for its expression.

Feed Conversion Ratio: The term "feed conversion ratio" the amount of feed fed to an animal to increase the weight of the animal by a specified amount. An improved feed conversion ratio means a lower feed conversion ratio. By "lower feed conversion ratio" or "improved feed conversion ratio" it is meant that the use of a feed additive composition in feed results in a lower amount of feed being required to be fed to an animal to increase the weight of the animal by a specified amount compared to the amount of feed required to increase the weight of the animal by the same amount when the feed does not comprise said feed additive composition.

Feed efficiency: The term "feed efficiency" means the amount of weight gain per unit of feed when the animal is fed ad-libitum or a specified amount of food during a period of time. By "increased feed efficiency" it is meant that the use of a feed additive composition according the

present invention in feed results in an increased weight gain per unit of feed intake compared with an animal fed without said feed additive composition being present.

Fragment: The term "fragment" means a polypeptide having one or more (*e.g.*, several) amino acids absent from the amino and/or carboxyl terminus of a mature polypeptide or domain; wherein the fragment has alpha-galactosidase activity.

In one aspect, the fragment comprises at least 90% of the length of the mature polypeptide, such as at least 648 amino acids of SEQ ID NO: 2 or at least 648 amino acids of SEQ ID NO: 3. In another aspect, the fragment comprises at least 92% of the length of the mature polypeptide, such as at least 663 amino acids of SEQ ID NO: 2 or at least 663 amino acids of SEQ ID NO: 3. In another aspect, the fragment comprises at least 94% of the length of the mature polypeptide, such as at least 677 amino acids of SEQ ID NO: 2 or at least 677 amino acids of SEQ ID NO: 3.

In another aspect, the fragment comprises at least 96% of the length of the mature polypeptide, such as at least 692 amino acids of SEQ ID NO: 2 or at least 692 amino acids of SEQ ID NO: 3. In another aspect, the fragment comprises at least 98% of the length of the mature polypeptide, such as at least 706 amino acids of SEQ ID NO: 2 or at least 706 amino acids of SEQ ID NO: 3. In another aspect, the fragment comprises at least 99% of the length of the mature polypeptide, such as at least 713 amino acids of SEQ ID NO: 2 or at least 713 amino acids of SEQ ID NO: 3.

Host cell: The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, or the like with a nucleic acid construct or expression vector comprising a polynucleotide of the present invention. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

Isolated: The term "isolated" means a substance in a form or environment that does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally occurring substance, (2) any substance including, but not limited to, any enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified by the hand of man relative to that substance found in nature; or (4) any substance modified by increasing the amount of the substance relative to other components with which it is naturally associated (*e.g.*, recombinant production in a host cell; multiple copies of a gene encoding the substance; and use of a stronger promoter than the promoter naturally associated with the gene encoding the substance).

Mature polypeptide: The term "mature polypeptide" means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. In one aspect, the mature polypeptide

is amino acids 1 to 721 of SEQ ID NO: 2. In another aspect, the mature polypeptide is amino acids 1 to 721 of SEQ ID NO: 3.

It is known in the art that a host cell may produce a mixture of two or more different mature polypeptides (*i.e.*, with a different C-terminal and/or N-terminal amino acid) expressed by the same polynucleotide. It is also known in the art that different host cells process polypeptides differently, and thus, one host cell expressing a polynucleotide may produce a different mature polypeptide (*e.g.*, having a different C-terminal and/or N-terminal amino acid) as compared to another host cell expressing the same polynucleotide.

Mature polypeptide coding sequence: The term "mature polypeptide coding sequence" means a polynucleotide that encodes a mature polypeptide having alpha-galactosidase activity. In one aspect, the mature polypeptide coding sequence is the joined sequence of nucleotides 1 to 82, nucleotides 143 to 746 and nucleotides 822 to 2361 of SEQ ID NO: 1.

Nucleic acid construct: The term "nucleic acid construct" means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic, which comprises one or more control sequences.

Nutrient Digestibility: The term "nutrient digestibility" means the fraction of a nutrient that disappears from the gastro-intestinal tract or a specified segment of the gastro-intestinal tract, *e.g.* the small intestine. Nutrient digestibility may be measured as the difference between what is administered to the subject and what comes out in the faeces of the subject, or between what is administered to the subject and what remains in the digesta on a specified segment of the gastro intestinal tract, *e.g.* the ileum.

Nutrient digestibility as used herein may be measured by the difference between the intake of a nutrient and the excreted nutrient by means of the total collection of excreta during a period of time; or with the use of an inert marker that is not absorbed by the animal, and allows the researcher calculating the amount of nutrient that disappeared in the entire gastro-intestinal tract or a segment of the gastro-intestinal tract. Such an inert marker may be titanium dioxide, chromic oxide or acid insoluble ash. Digestibility may be expressed as a percentage of the nutrient in the feed, or as mass units of digestible nutrient per mass units of nutrient in the feed. Nutrient digestibility as used herein encompasses starch digestibility, fat digestibility, protein digestibility, and amino acid digestibility.

Energy digestibility as used herein means the gross energy of the feed consumed minus the gross energy of the faeces or the gross energy of the feed consumed minus the gross energy of the remaining digesta on a specified segment of the gastro-intestinal tract of the animal, *e.g.* the ileum. Metabolizable energy as used herein refers to apparent metabolizable energy and means the gross energy of the feed consumed minus the gross energy contained in the faeces, urine, and gaseous products of digestion. Energy digestibility and metabolizable energy may be

measured as the difference between the intake of gross energy and the gross energy excreted in the faeces or the digesta present in specified segment of the gastro-intestinal tract using the same methods to measure the digestibility of nutrients, with appropriate corrections for nitrogen excretion to calculate metabolizable energy of feed.

5 **Operably linked:** The term “operably linked” means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs expression of the coding sequence.

Release x g galactose per kg soybean meal: The term “release x g galactose per kg soybean meal” means the amount of galactose in grams which is released into the supernatant after soybean meal has been incubation with an enzyme. For the purpose of the present invention, the release of galactose per kg soybean meal may be determined when performed under the reaction conditions 20 mg polypeptide per kg soybean meal in 10% w/v 0.1M citric acid-phosphate buffer pH 6.5 incubating at 40°C for 2 hours as described in the galactose assay herein.

 In a more detailed embodiment, a 10 w/v% slurry of soybean meal is prepared from soybean meal milled to a 0.5 mm particle size and 0.1 M citric acid-phosphate buffer, pH 6.5±0.05. The incubation vessels with the 10 w/v% slurry of soybean meal is heated to a stable temperature of 40±2 °C while stirring. When a stable temperature had been achieved, the six D-(+)-galactose standards are added to the incubation vessels to in-vessel concentrations of 5, 2.5, 1.25, 0.625, 0.313 and 0.157 mg galactose per mL incubation volume. Each standard is incubated in duplicates. The diluted enzymes are then added to their respective incubation vessels in the volumes required to reach their desired concentrations (in mg EP/kg soybean meal). Each enzyme treatment is incubated in triplicates. Additionally, two times three incubation vessels are included without standards or enzyme treatments as blank treatments to obtain the baseline galactose concentration in the soybean meal slurry. The incubation vessels are incubated at 40±2 °C, while stirring for 2 hours. After incubation the vessels are centrifuged at 1500 g at 5 °C for 15 minutes. The supernatants are then analyzed in an assay based on the Raffinose/Galactose kit from Megazyme (product name K-RAFGA) and the concentration of galactose is then calculated as described in the galactose assay herein.

Plant based material: The term “plant based material” means that the plant based material is from the taxonomic subclass *rosids*. In one aspect, the plant based material is from the taxonomic order *Fabales*, such as the family *Fabaceae*, preferably the subfamilies *Caesalpinioideae* or *Mimosoideae* or *Papilionoideae*, or more preferably from the tribes *Phaseoleae*, *Cicereae*, *Genisteae*, *Fabeae*, *Dalbergieae* or *Phaseoleae*. In one aspect, the plant based material is from the taxonomic order *Brassicales*, such as the family *Brassicaceae*, preferably the tribe *Brassiceae*, more preferably the family *Brassica*.

 In particular embodiments, the plant based material is soybean, wild soybean, beans, lupin, tepary bean, scarlet runner bean, slimjim bean, lima bean, French bean, Broad bean (fava

bean), chickpea, lentil, peanut, Spanish peanut, canola, rapeseed (oilseed rape) or pea or in a processed form such as soybean meal, full fat soy bean meal, soy protein concentrate (SPC), fermented soybean meal (FSBM) or any combination thereof. In a preferred embodiment, the plant based material is soybean or soybean meal.

5 **Sequence identity:** The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter “sequence identity”.

For purposes of the present invention, the degree of sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 3.0.0 or later. Version 6.1.0 was used. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labelled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

$$\text{(Identical Residues x 100)} / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

For purposes of the present invention, the degree of sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 3.0.0 or later. Version 6.1.0 was used. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labelled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

25
$$\text{(Identical Deoxyribonucleotides x 100)} / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

Stringency conditions: The different stringency conditions are defined as follows.

The term “very low stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2.0X SSC, 0.2% SDS at 60°C.

The term “low stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2.0X SSC, 0.2% SDS at 65°C.

The term "medium stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 5 15 minutes using 1.0X SSC, 0.2% SDS at 65°C.

The term "medium-high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed 10 three times each for 15 minutes using 1.0X SSC, 0.2% SDS at 70°C.

The term "high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 15 minutes using 0.5X SSC, 0.2% SDS at 70°C.

The term "very high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 20 15 minutes using 0.5X SSC, 0.2% SDS at 75°C.

Subsequence: The term "subsequence" means a polynucleotide having one or more (*e.g.*, several) nucleotides absent from the 5' and/or 3' end of a mature polypeptide coding sequence; wherein the subsequence encodes a fragment having alpha-galactosidase activity.

Substantially pure polypeptide: The term "substantially pure polypeptide" means a 25 preparation that contains at most 10%, at most 8%, at most 6%, at most 5%, at most 4%, at most 3%, at most 2%, at most 1%, and at most 0.5% by weight of other polypeptide material with which it is natively or recombinantly associated. Preferably, the polypeptide is at least 92% pure, *e.g.*, at least 94% pure, at least 95% pure, at least 96% pure, at least 97% pure, at least 98% pure, at least 99%, at least 99.5% pure, and 100% pure by weight of the total polypeptide material present 30 in the preparation. The polypeptides of the present invention are preferably in a substantially pure form. This can be accomplished, for example, by preparing the polypeptide by well-known recombinant methods or by classical purification methods.

Variant: The term "variant" means a polypeptide having alpha-galactosidase activity comprising an alteration, *i.e.*, a substitution, insertion, and/or deletion of one or more (several) 35 amino acid residues at one or more (several) positions. A substitution means a replacement of an amino acid occupying a position with a different amino acid; a deletion means removal of an

amino acid occupying a position; and an insertion means adding 1-3 amino acids adjacent to an amino acid occupying a position.

In one aspect, the variants of the present invention have at least 40%, such as at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 100% of the alpha-galactosidase activity of the polypeptide of SEQ ID NO: 3 when using 4-nitrophenyl α -D-galactopyranoside as substrate as described herein.

In one aspect, the variant has improved gastric stability compared to control (wherein control is defined as SEQ ID NO: 4). In an embodiment, the gastric stability is measured as the half-life at 40°C pH3 with 0.1 mg/ml pepsin. In an embodiment, gastric stability is measured as described in example 4 herein. In a further embodiment, the variant has a half-life of at least 90 minutes, such as 2 hours, 3 hours or 4 hours.

In one aspect, the variant has increased alpha-galactosidase activity compared to control (wherein control is defined as SEQ ID NO: 4). In one embodiment, activity is measured using 4-nitrophenyl α -D-galactopyranoside as substrate. In one embodiment, activity is measured using 4-nitrophenyl α -D-galactopyranoside as substrate (1 mg/ml in 100 mM MES buffer pH 7.0 \pm 0.05, prepared immediately before use) as substrate by measuring the absorbance of released pNP in the buffer at 405 nm for 5 minutes as function of time at room temperature (typically 23°C). In an embodiment, activity is measured as described in example 3 herein. In an embodiment, the variant has an activity of at least 100000 (mOD/min)/(mg/ml), such as at least 150000, at least 200000, at least 250000, or at least 300000 (mOD/min)/(mg/ml) using 4-nitrophenyl α -D-galactopyranoside as substrate.

Detailed Description of the Invention

The inventors have found that certain alpha-galactosidases from glycoside hydrolase family 36 (herein referred to as GH36) are surprisingly good at degrading the raffinose family of oligosaccharides (RFOs), such as the trisaccharide raffinose, the tetrasaccharide stachyose, and the pentasaccharide verbascose. These RFOs are typically found in plants from the taxonomic subclass *rosids*, specifically the subfamily *Papilionoideae*, such as in soy beans, chickpea, beans, lupin, lentil, peanut and peas or the tribe *Brassicaceae*, such as in canola or rapeseed.

Efficient alpha-galactosidases are required to effectively degrade RFO's. However, the alpha-galactoside also needs to survive passage through the stomach of the animal where there is a low pH and proteases such as pepsin, before it can get to work in the intestine. If the alpha-galactosidase is degraded or denatured whilst passing through the stomach then it will not be present to work in the intestine of the animal.

The degradation of RFO's can be measured as the amount of galactose released into the supernatant when e.g. soybean meal is treated with an alpha-galactosidase. Increased amounts of solubilisation will result in more galactose being released which can be detected using e.g. the

Galactose SBM Assay method as described herein. Thus the invention relates to an acid stable alpha-galactosidase which is also highly efficient at releasing galactose from plant material, such as soybean meal.

Animal feed and animal feed additives

5 In a first aspect, the invention relates to animal feed additives comprising one or more GH36 polypeptides having alpha-galactosidase activity, wherein the polypeptide having alpha-galactosidase activity has at least 80%, *e.g.*, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the
10 mature polypeptide of SEQ ID NO: 2. In one embodiment, the polypeptides differ by up to 50 amino acids, *e.g.*, between 1 and 50 amino acids, such as 1-45, 1-40, 1-35, 1-30, 1-25, 1-20, 1-15, 1-10 or 1-5 amino acids, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 amino acids from the mature polypeptide of SEQ ID NO: 2.

15 In a continuation of the first aspect, the invention relates to animal feed additives comprising one or more GH36 polypeptides having alpha-galactosidase activity, wherein the polypeptide having alpha-galactosidase activity having at least 82%, *e.g.*, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%
20 sequence identity to SEQ ID NO: 3. In one embodiment, the polypeptides differ by up to 50 amino acids, *e.g.*, between 1 and 50 amino acids, such as 1-45, 1-40, 1-35, 1-30, 1-25, 1-20, 1-15, 1-10 or 1-5 amino acids, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 amino acids from SEQ ID NO: 3.

25 In one embodiment, the polypeptide preferably comprises or consists of the amino acid sequence of SEQ ID NO: 3 or an allelic variant thereof; comprises the amino acid sequence of SEQ ID NO: 3 and a N-terminal and/or C-terminal His-tag and/or HQ-tag; or is a fragment thereof having alpha-galactosidase activity and having at least 90% such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%
30 of the length of the mature polypeptide. In another embodiment, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 3. In another embodiment, the polypeptide comprises or consists of amino acids 1 to 721 of SEQ ID NO: 2. In another embodiment, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 2. In another embodiment, the polypeptide comprises or consists of amino acids 1 to 721 of SEQ ID NO: 3. In
35 an embodiment, the polypeptide has been isolated.

 In a continuation of the first aspect, the invention relates to animal feed additives comprising one or more GH36 polypeptides having alpha-galactosidase activity, wherein the

polypeptide having alpha-galactosidase activity encoded by a polynucleotide that hybridizes under high or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the cDNA sequence thereof, or (iii) the full-length complement of (i) or (ii) (Sambrook *et al.*, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York). In an embodiment, the polypeptide has been isolated.

In a continuation of the first aspect, the invention relates to animal feed additives comprising one or more GH36 polypeptides having alpha-galactosidase activity, wherein the polypeptide having alpha-galactosidase activity encoded by a polynucleotide having a sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1 of at least 80%, *e.g.*, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. In a further embodiment, the polypeptide has been isolated.

In one embodiment of the first aspect of the invention, the polypeptide has improved gastric stability compared to control (wherein control is defined as SEQ ID NO: 4). In an embodiment, the gastric stability is measured as the half-life at 40°C pH3 with 0.1 mg/ml pepsin. In an embodiment, gastric stability is measured as described in example 4 herein. In a further embodiment, the polypeptide has a half-life of at least 90 minutes, such as 2 hours, 3 hours or 4 hours.

In one embodiment of the first aspect of the invention, the polypeptide has increased alpha-galactosidase activity compared to control (wherein control is defined as SEQ ID NO: 4). In one embodiment, activity is measured using 4-nitrophenyl α -D-galactopyranoside as substrate. In one embodiment, activity is measured using 4-nitrophenyl α -D-galactopyranoside as substrate (1 mg/ml in 100 mM MES buffer pH 7.0 \pm 0.05, prepared immediately before use) as substrate by measuring the absorbance of released pNP in the buffer at 405 nm for 5 minutes as function of time at room temperature (typically 23°C). In an embodiment, activity is measured as described in example 3 herein. In an embodiment, the polypeptide has an activity of at least 100000 (mOD/min)/(mg/ml), such as at least 150000, at least 200000, at least 250000, or at least 300000 (mOD/min)/(mg/ml) using 4-nitrophenyl α -D-galactopyranoside as substrate. In an embodiment, the polypeptide has at least 40%, such as at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 100% of the alpha-galactosidase activity of the polypeptide of SEQ ID NO: 3 (as determined using 4-nitrophenyl α -D-galactopyranoside as substrate).

In a continuation of the first aspect, the invention relates to animal feed additives comprising variants of SEQ ID NO: 3, wherein the variant of SEQ ID NO: 3 has alpha-galactosidase activity and comprises one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof at one or more (*e.g.*, several) positions. In an embodiment, the number of positions comprising one or

more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in SEQ ID NO: 3 is not more than 50, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50. In another embodiment, the number of positions comprising one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in SEQ ID NO: 3 is between 1 and 45, such as 1-40, 1-35, 1-30, 1-25, 1-20, 1-15, 1-10 or 1-5 positions. In an embodiment, the number of positions comprising one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in SEQ ID NO: 3 is not more than 10, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. In another embodiment, the number of substitutions and/or deletions and/or insertions in SEQ ID NO: 3 is not more than 10, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. In a further embodiment, the number of substitutions in SEQ ID NO: 3 is not more than 10, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. In a further embodiment, the number of conservative substitutions in SEQ ID NO: 3 is not more than 10, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

The amino acid changes may be of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of 1-30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the groups of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. Common substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly. Other examples of conservative substitutions are G to A; A to G, S; V to I, L, A, T, S; I to V, L, M; L to I, M, V; M to L, I, V; P to A, S, N; F to Y, W, H; Y to F, W, H; W to Y, F, H; R to K, E, D; K to R, E, D; H to Q, N, S; D to N, E, K, R, Q; E to Q, D, K, R, N; S to T, A; T to S, V, A; C to S, T, A; N to D, Q, H, S; Q to E, N, H, K, R.

Essential amino acids in a polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for

alpha-galactosidase activity to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron
5 diffraction, or photoaffinity labelling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309: 59-64. The identity of essential amino acids can also be inferred from an alignment with a related polypeptide.

Single or multiple amino acid substitutions, deletions, and/or insertions can be made and
10 tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage display (e.g., Lowman *et al.*, 1991, *Biochemistry* 30: 10832-10837; U.S. Patent No. 5,223,409;
15 WO 92/06204), and region-directed mutagenesis (Derbyshire *et al.*, 1986, *Gene* 46: 145; Ner *et al.*, 1988, *DNA* 7: 127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness *et al.*, 1999, *Nature Biotechnology* 17: 893-896). Mutagenized DNA molecules that encode
20 active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide.

The polypeptide may be a hybrid polypeptide in which a region of one polypeptide is fused at the N-terminus or the C-terminus of a region of another polypeptide.

25 The polypeptide may be a fusion polypeptide or cleavable fusion polypeptide in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide of the present invention. A fusion polypeptide is produced by fusing a polynucleotide encoding another polypeptide to a polynucleotide of the present invention. Techniques for producing fusion polypeptides are known in the art, and include ligating the coding sequences encoding the
30 polypeptides so that they are in frame and that expression of the fusion polypeptide is under control of the same promoter(s) and terminator. Fusion polypeptides may also be constructed using intein technology in which fusion polypeptides are created post-translationally (Cooper *et al.*, 1993, *EMBO J.* 12: 2575-2583; Dawson *et al.*, 1994, *Science* 266: 776-779).

A fusion polypeptide can further comprise a cleavage site between the two polypeptides.
35 Upon secretion of the fusion protein, the site is cleaved releasing the two polypeptides. Examples of cleavage sites include, but are not limited to, the sites disclosed in Martin *et al.*, 2003, *J. Ind. Microbiol. Biotechnol.* 3: 568-576; Svetina *et al.*, 2000, *J. Biotechnol.* 76: 245-251; Rasmussen-

Wilson *et al.*, 1997, *Appl. Environ. Microbiol.* 63: 3488-3493; Ward *et al.*, 1995, *Biotechnology* 13: 498-503; and Contreras *et al.*, 1991, *Biotechnology* 9: 378-381; Eaton *et al.*, 1986, *Biochemistry* 25: 505-512; Collins-Racie *et al.*, 1995, *Biotechnology* 13: 982-987; Carter *et al.*, 1989, *Proteins: Structure, Function, and Genetics* 6: 240-248; and Stevens, 2003, *Drug Discovery World* 4: 35-48.

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In one embodiment of the first aspect of the invention, the variant has improved gastric stability compared to control (wherein control is defined as SEQ ID NO: 4). In an embodiment, the gastric stability is measured as the half-life at 40°C pH3 with 0.1 mg/ml pepsin. In an embodiment, gastric stability is measured as described in example 4 herein. In a further embodiment, the variant has a half-life of at least 90 minutes, such as 2 hours, 3 hours or 4 hours.

In one embodiment of the first aspect of the invention, the variant has increased alpha-galactosidase activity compared to control (wherein control is defined as SEQ ID NO: 4). In one embodiment, activity is measured using 4-nitrophenyl α -D-galactopyranoside as substrate. In one embodiment, activity is measured using 4-nitrophenyl α -D-galactopyranoside as substrate (1 mg/ml in 100 mM MES buffer pH 7.0 \pm 0.05, prepared immediately before use) as substrate by measuring the absorbance of released pNP in the buffer at 405 nm for 5 minutes as function of time at room temperature (typically 23°C). In an embodiment, activity is measured as described in example 3 herein. In an embodiment, the variant has an activity of at least 100000 (mOD/min)/(mg/ml), such as at least 150000, at least 200000, at least 250000, or at least 300000 (mOD/min)/(mg/ml) using 4-nitrophenyl α -D-galactopyranoside as substrate. In an embodiment, the variant has at least 40%, such as at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 100% of the alpha-galactosidase activity of the variant of SEQ ID NO: 3 (as determined using 4-nitrophenyl α -D-galactopyranoside as substrate).

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In an embodiment, the plant based material is from the taxonomic subclass *rosids*. In one aspect, the plant based material is from the taxonomic order *Fabales*, such as the family *Fabaceae*, preferably the subfamilies *Caesalpinioideae* or *Mimosoideae* or *Papilionoideae*, or more preferably from the tribes *Phaseoleae*, *Cicereae*, *Genisteae*, *Fabeae*, *Dalbergieae* or *Phaseoleae*. In one aspect, the plant based material is from the taxonomic order *Brassicales*, such as the family *Brassicaceae*, preferably the tribe *Brassiceae*, more preferably the family *Brassica*.

In particular embodiments, the plant based material is soybean, wild soybean, beans, lupin, tepary bean, scarlet runner bean, slimjim bean, lima bean, French bean, Broad bean (fava bean), chickpea, lentil, peanut, Spanish peanut, canola, rapeseed (oilseed rape) or pea or in a processed form such as soybean meal, full fat soy bean meal, soy protein concentrate (SPC), fermented soybean meal (FSBM) or any combination thereof. In a preferred embodiment, the plant based material is soybean or soybean meal.

Polypeptides Having Alpha-galactosidase Activity

In a second aspect, the invention relates to polypeptides having alpha-galactosidase activity having at least 96.5%, *e.g.*, at least 97%, at least 97.5%, at least 98%, at least 98.5%, at least 99%, at least 99.5%, or 100% sequence identity to the mature polypeptide of SEQ ID NO: 2. In one embodiment, the polypeptides differ by up to 27 amino acids, *e.g.*, between 1 and 27 amino acids, such as 1-25, 1-20, 1-15, 1-10 or 1-5 amino acids, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 or 27 amino acids from the mature polypeptide of SEQ ID NO: 2.

In an embodiment, the polypeptide releases at least 14g, such as at least 14.5g, at least 15g, at least 16g, at least 17g, at least 18g, at least 19g, at least 20g or at least 22g galactose per kg soybean meal when performed under the reaction conditions 20 mg polypeptide per kg soybean meal in 10% w/v 0.1M citric acid-phosphate buffer pH 6.5 incubating at 40°C for 2 hours.

In a continuation of the second aspect, the invention relates to polypeptides having alpha-galactosidase activity having at least 96.5%, *e.g.*, at least 97%, at least 97.5%, at least 98%, at least 98.5%, at least 99%, at least 99.5%, or 100% sequence identity to SEQ ID NO: 3. In one embodiment, the polypeptides differ by up to 27 amino acids, *e.g.*, between 1 and 27 amino acids, such as 1-25, 1-20, 1-15, 1-10 or 1-5 amino acids, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 or 27 amino acids from the mature polypeptide of SEQ ID NO: 3.

In one embodiment, the polypeptide preferably comprises or consists of the amino acid sequence of SEQ ID NO: 3 or an allelic variant thereof; comprises the amino acid sequence of SEQ ID NO: 3 and a N-terminal and/or C-terminal His-tag and/or HQ-tag; or is a fragment thereof having alpha-galactosidase activity and having at least 90% such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% of the length of the mature polypeptide. In another embodiment, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 3. In another embodiment, the polypeptide comprises or consists of amino acids 1 to 721 of SEQ ID NO: 2. In another embodiment, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 2. In another embodiment, the polypeptide comprises or consists of amino acids 1 to 721 of SEQ ID NO: 3. In an embodiment, the polypeptide has been isolated.

In a continuation of the second aspect, the invention relates to a polypeptide having alpha-galactosidase activity encoded by a polynucleotide having a sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1 of at least 96.5%, *e.g.*, at least 97%, at least 97.5%, at least 98%, at least 98.5%, at least 99%, at least 99.5%, or 100%. In a further embodiment, the polypeptide has been isolated.

In a continuation of the second aspect, the invention relates to variants of SEQ ID NO: 3 having alpha-galactosidase activity comprising one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof at one or more (*e.g.*, several) positions. In an embodiment, the number of positions comprising one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in SEQ ID NO: 3 is not more than 27, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 or 27. In another embodiment, the number of positions comprising one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in SEQ ID NO: 3 is between 1 and 25, such as 1-20, 1-15, 1-10 or 1-5 positions. In an embodiment, the number of positions comprising one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in SEQ ID NO: 3 is not more than 10, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. In another embodiment, the number of substitutions and/or deletions and/or insertions in SEQ ID NO: 3 is not more than 10, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. In a further embodiment, the number of substitutions in SEQ ID NO: 3 is not more than 10, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. In a further embodiment, the number of conservative substitutions in SEQ ID NO: 3 is not more than 10, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Examples of amino acid changes, conservative substitutions and fusion peptides are described in the first aspect herein.

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Granules Comprising Polypeptides Having Alpha-galactosidase Activity

In a third aspect, the invention relates to a granule comprising a GH36 polypeptide having alpha-galactosidase activity, wherein the polypeptide is selected from the group consisting of:

- 25 (a) a polypeptide having at least 80%, *e.g.*, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the polypeptide of SEQ ID NO: 3;
- 30 (b) a variant of SEQ ID NO: 3, wherein the variant has alpha-galactosidase activity and comprises one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 positions;
- 35 (c) a polypeptide comprising the polypeptide of (a) or (b) and a N-terminal and/or C-terminal His-tag and/or HQ-tag;

- (d) a polypeptide comprising the polypeptide of (a) or (b) and a N-terminal and/or C-terminal extension of up to 10 amino acids, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids; and
- (e) a fragment of the polypeptide of (a) or (b) having alpha-galactosidase activity and having at least 90% of the length of the mature polypeptide.

In one embodiment, the granule comprises a core particle and one or more coatings. In a preferred embodiment, the coating comprises salt and/or wax and/or flour. Preferred formulations are disclosed in the formulation section below.

In one embodiment of the third aspect of the invention, the polypeptide has improved gastric stability compared to control (wherein control is defined as SEQ ID NO: 4). In an embodiment, the gastric stability is measured as the half-life at 40°C pH3 with 0.1 mg/ml pepsin. In an embodiment, gastric stability is measured as described in example 4 herein. In a further embodiment, the polypeptide has a half-life of at least 90 minutes, such as 2 hours, 3 hours or 4 hours.

In one embodiment of the third aspect of the invention, the polypeptide has increased alpha-galactosidase activity compared to control (wherein control is defined as SEQ ID NO: 4). In one embodiment, activity is measured using 4-nitrophenyl α -D-galactopyranoside as substrate. In one embodiment, activity is measured using 4-nitrophenyl α -D-galactopyranoside as substrate (1 mg/ml in 100 mM MES buffer pH 7.0 \pm 0.05, prepared immediately before use) as substrate by measuring the absorbance of released pNP in the buffer at 405 nm for 5 minutes as function of time at room temperature (typically 23°C). In an embodiment, activity is measured as described in example 3 herein. In an embodiment, the polypeptide has an activity of at least 100000 (mOD/min)/(mg/ml), such as at least 150000, at least 200000, at least 250000, or at least 300000 (mOD/min)/(mg/ml) using 4-nitrophenyl α -D-galactopyranoside as substrate. In an embodiment, the polypeptide has at least 40%, such as at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 100% of the alpha-galactosidase activity of the polypeptide of SEQ ID NO: 3 (as determined using 4-nitrophenyl α -D-galactopyranoside as substrate).

In one embodiment of the third aspect of the invention, the composition comprises at least 0.01 mg of polypeptide (enzyme protein) per kilogram of composition, such as at least 0.02 mg, 0.05 mg, 0.10 mg, 0.2 mg, 0.5 mg, 1.0 mg, 2 mg, 5 mg, 10 mg, 20 mg, 50mg, 100 mg, 200 mg, 500mg, 1.0 g, 2.5 g, 5 g, 7.5 g, 10 g, 25 g, 50 g, 75 g or 100 g per kilogram of composition. In one embodiment, the composition comprises at most 250g of polypeptide per kilogram of composition, such as at most 150 g, 100 g, 50 g, 40 g, 30 g, 20 g, 10 g, 7.5 g, 5 g, 2.5 g, 1.0 g, 750 mg, 500 mg, 250 mg, 100 mg, 50 mg, 25 mg, 10 mg, 5 mg, 2.5 mg or 1 mg per kilogram of composition. In one embodiment, the composition comprises between 0.01 mg and 250g of

polypeptide (enzyme protein) per kilogram of composition, such as between 0.02 mg, 0.05 mg, 0.10 mg, 0.2 mg, 0.5 mg, 1.0 mg, 2 mg, 5 mg, 10 mg, 20 mg, 50mg, 100 mg, 200 mg, 500mg, 1.0 g, 2.5 g, 5 g, 7.5 g, 10 g, 25 g, 50 g, 75 g or 100 g per kilogram of composition and 150 g, 100 g, 50 g, 40 g, 30 g, 20 g, 10 g, 7.5 g, 5 g, 2.5 g, 1.0 g, 750 mg, 500 mg, 250 mg, 100 mg, 50 mg, 25 mg, 10 mg, 5 mg, 2.5 mg or 1 mg per kilogram of composition, or any combination thereof.

In one embodiment of the third aspect of the invention, the granule comprises one or more formulating agents (such as those described herein), preferably a formulating agent selected from the list consisting of glycerol, ethylene glycol, 1, 2-propylene glycol or 1, 3-propylene glycol, sodium chloride, sodium benzoate, potassium sorbate, sodium sulfate, potassium sulfate, magnesium sulfate, sodium thiosulfate, calcium carbonate, sodium citrate, dextrin, glucose, sucrose, sorbitol, lactose, starch, kaolin, maltodextrin, cyclodextrin, wheat, PVA, acetate, phosphate and cellulose, preferably selected from the list consisting of 1, 2-propylene glycol, 1, 3-propylene glycol, sodium sulfate, dextrin, cellulose, sodium thiosulfate, kaolin and calcium carbonate.

In one embodiment of the third aspect of the invention, the granule comprises a core particle and one or more coatings. In a preferred embodiment, the coating comprises salt and/or wax and/or flour. Preferred formulations are disclosed in the formulation section below.

In one embodiment of the third aspect of the invention, the granule comprises one or more additional enzymes. The one or more additional enzymes is preferably selected from the group consisting of acetylxylan esterase, acylglycerol lipase, amylase, alpha-amylase, beta-amylase, arabinofuranosidase, cellobiohydrolases, cellulase, feruloyl esterase, galactanase, alpha-galactosidase, beta-galactosidase, beta-glucanase, beta-glucosidase, lysophospholipase, lysozyme, alpha-mannosidase, beta-mannosidase (mannanase), phytase, phospholipase A1, phospholipase A2, phospholipase D, protease, pullulanase, pectinesterase, triacylglycerol lipase, xylanase, beta-xylosidase or any combination thereof.

In one embodiment of the third aspect of the invention, the granule comprises one or more probiotics. The one or more probiotics is preferably selected from the group consisting of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus pumilus*, *Bacillus polymyxa*, *Bacillus megaterium*, *Bacillus coagulans*, *Bacillus circulans*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, *Bifidobacterium sp.*, *Carnobacterium sp.*, *Clostridium butyricum*, *Clostridium sp.*, *Enterococcus faecium*, *Enterococcus sp.*, *Lactobacillus sp.*, *Lactobacillus acidophilus*, *Lactobacillus farciminus*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactococcus lactis*, *Lactococcus sp.*, *Leuconostoc sp.*, *Megasphaera elsdenii*, *Megasphaera sp.*, *Pediococcus acidilactici*, *Pediococcus sp.*, *Propionibacterium thoenii*, *Propionibacterium sp.* and *Streptococcus sp.* or any combination thereof.

Liquid formulations comprising polypeptides Having Alpha-galactosidase Activity

In a fourth aspect, the invention relates to a liquid formulation comprising one or more GH36 polypeptides having alpha-galactosidase activity, wherein the liquid formulation comprises:

- 5 (A) 0.001% to 25% w/w of polypeptide having alpha-galactosidase activity wherein the polypeptide having alpha-galactosidase activity is selected from the group consisting of:
- 10 (a) a polypeptide having at least 80%, e.g., at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the polypeptide of SEQ ID NO: 3;
 - 15 (b) a variant of SEQ ID NO: 3, wherein the variant has alpha-galactosidase activity and comprises one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 positions;
 - (c) a polypeptide comprising the polypeptide of (a) or (b) and a N-terminal and/or C-terminal His-tag and/or HQ-tag;
 - (d) a polypeptide comprising the polypeptide of (a) or (b) and a N-terminal and/or C-terminal extension of up to 10 amino acids, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids; and
 - 20 (e) a fragment of the polypeptide of (a) or (b) having alpha-galactosidase activity and having at least 90% of the length of the mature polypeptide; and
- (B) water.

25 In one embodiment, the liquid formulation comprises 20%-80% polyol. In one embodiment, the liquid formulation comprises 0.001% to 2.0% w/w preservative.

In one embodiment, the invention relates to a liquid formulation comprising one or more GH36 polypeptides having alpha-galactosidase activity, wherein the liquid formulation comprises:

- 30 (A) 0.001% to 25% w/w of polypeptide having alpha-galactosidase activity wherein the polypeptide having alpha-galactosidase activity is selected from the group consisting of:
- 35 (a) a polypeptide having at least 80%, e.g., at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the polypeptide of SEQ ID NO: 3;
 - (b) a variant of SEQ ID NO: 3, wherein the variant has alpha-galactosidase activity and comprises one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination

thereof in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 positions;

(c) a polypeptide comprising the polypeptide of (a) or (b) and a N-terminal and/or C-terminal His-tag and/or HQ-tag;

(d) a polypeptide comprising the polypeptide of (a) or (b) and a N-terminal and/or C-terminal extension of up to 10 amino acids, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids; and

(e) a fragment of the polypeptide of (a) or (b) having alpha-galactosidase activity and having at least 90% of the length of the mature polypeptide; and

(B) 20% to 80% w/w of polyol;

(C) 0.001% to 2.0% w/w preservative; and

(D) water.

In one embodiment of the fourth aspect of the invention, the polypeptide has improved gastric stability compared to control (wherein control is defined as SEQ ID NO: 4). In an embodiment, the gastric stability is measured as the half-life at 40°C pH3 with 0.1 mg/ml pepsin. In an embodiment, gastric stability is measured as described in example 4 herein. In a further embodiment, the polypeptide has a half-life of at least 90 minutes, such as 2 hours, 3 hours or 4 hours.

In one embodiment of the fourth aspect of the invention, the polypeptide has increased alpha-galactosidase activity compared to control (wherein control is defined as SEQ ID NO: 4). In one embodiment, activity is measured using 4-nitrophenyl α -D-galactopyranoside as substrate. In one embodiment, activity is measured using 4-nitrophenyl α -D-galactopyranoside as substrate (1 mg/ml in 100 mM MES buffer pH 7.0 \pm 0.05, prepared immediately before use) as substrate by measuring the absorbance of released pNP in the buffer at 405 nm for 5 minutes as function of time at room temperature (typically 23°C). In an embodiment, activity is measured as described in example 3 herein. In an embodiment, the polypeptide has an activity of at least 100000 (mOD/min)/(mg/ml), such as at least 150000, at least 200000, at least 250000, or at least 300000 (mOD/min)/(mg/ml) using 4-nitrophenyl α -D-galactopyranoside as substrate. In an embodiment, the polypeptide has at least 40%, such as at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 100% of the alpha-galactosidase activity of the polypeptide of SEQ ID NO: 3 (as determined using 4-nitrophenyl α -D-galactopyranoside as substrate).

In one embodiment of the fourth aspect of the invention, the liquid formulation comprises one or more formulating agents (such as those described herein), preferably a formulating agent selected from the list consisting of glycerol, ethylene glycol, 1, 2-propylene glycol or 1, 3-

propylene glycol, sodium chloride, sodium benzoate, potassium sorbate, sodium sulfate, potassium sulfate, magnesium sulfate, sodium thiosulfate, calcium carbonate, sodium citrate, dextrin, glucose, sucrose, sorbitol, lactose, starch, PVA, acetate and phosphate, preferably selected from the list consisting of 1, 2-propylene glycol, 1, 3-propylene glycol, sodium sulfate, 5 dextrin, cellulose, sodium thiosulfate, kaolin and calcium carbonate.

In one embodiment of the fourth aspect of the invention, the liquid formulation comprises one or more polyols, preferably a polyol selected from the group consisting of glycerol, sorbitol, propylene glycol (MPG), ethylene glycol, diethylene glycol, triethylene glycol, 1, 2-propylene glycol or 1, 3-propylene glycol, dipropylene glycol, polyethylene glycol (PEG) having an average 10 molecular weight below about 600 and polypropylene glycol (PPG) having an average molecular weight below about 600, more preferably selected from the group consisting of glycerol, sorbitol and propylene glycol (MPG) or any combination thereof.

In one embodiment of the fourth aspect of the invention, the liquid formulation comprises 20%-80% polyol (i.e. total amount of polyol), preferably 25%-75% polyol, more preferably 30%- 15 70% polyol, more preferably 35%-65% polyol or most preferably 40%-60% polyol. In one embodiment, the liquid formulation comprises 20%-80% polyol, preferably 25%-75% polyol, more preferably 30%-70% polyol, more preferably 35%-65% polyol or most preferably 40%-60% polyol wherein the polyol is selected from the group consisting of glycerol, sorbitol, propylene glycol (MPG), ethylene glycol, diethylene glycol, triethylene glycol, 1, 2-propylene glycol or 1, 3- 20 propylene glycol, dipropylene glycol, polyethylene glycol (PEG) having an average molecular weight below about 600 and polypropylene glycol (PPG) having an average molecular weight below about 600. In one embodiment, the liquid formulation comprises 20%-80% polyol (i.e. total amount of polyol), preferably 25%-75% polyol, more preferably 30%-70% polyol, more preferably 35%-65% polyol or most preferably 40%-60% polyol wherein the polyol is selected from the group 25 consisting of glycerol, sorbitol and propylene glycol (MPG).

In one embodiment of the fourth aspect of the invention, the preservative is selected from the group consisting of sodium sorbate, potassium sorbate, sodium benzoate and potassium benzoate or any combination thereof. In one embodiment, the liquid formulation comprises 0.02% to 1.5% w/w preservative, more preferably 0.05% to 1.0% w/w preservative or most preferably 30 0.1% to 0.5% w/w preservative. In one embodiment, the liquid formulation comprises 0.001% to 2.0% w/w preservative (i.e. total amount of preservative), preferably 0.02% to 1.5% w/w preservative, more preferably 0.05% to 1.0% w/w preservative or most preferably 0.1% to 0.5% w/w preservative wherein the preservative is selected from the group consisting of sodium sorbate, potassium sorbate, sodium benzoate and potassium benzoate or any combination 35 thereof.

In one embodiment of the fourth aspect of the invention, the liquid formulation comprises 0.01% to 25% w/w polypeptide having alpha-galactosidase activity, preferably 0.05% to 20% w/w

polypeptide having alpha-galactosidase activity, more preferably 0.2% to 15% w/w polypeptide having alpha-galactosidase activity, more preferably 0.5% to 15% w/w polypeptide having alpha-galactosidase activity or most preferably 1.0% to 10% w/w polypeptide having alpha-galactosidase activity.

5 In one embodiment, the liquid formulation comprises one or more additional enzymes. The one or more additional enzymes is preferably selected from the group consisting of acetylxylylan esterase, acylglycerol lipase, amylase, alpha-amylase, beta-amylase, arabinofuranosidase, cellobiohydrolases, cellulase, feruloyl esterase, galactanase, alpha-galactosidase, beta-galactosidase, beta-glucanase, beta-glucosidase, lysophospholipase, lysozyme, alpha-mannosidase, beta-mannosidase (mannanase), phytase, phospholipase A1,
10 phospholipase A2, phospholipase D, protease, pullulanase, pectinesterase, triacylglycerol lipase, xylanase, beta-xylosidase or any combination thereof.

In one embodiment of the fourth aspect of the invention, the liquid formulation comprises one or more probiotics. The one or more probiotics is preferably selected from the group
15 consisting of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus pumilus*, *Bacillus polymyxa*, *Bacillus megaterium*, *Bacillus coagulans*, *Bacillus circulans*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, *Bifidobacterium sp.*, *Carnobacterium sp.*, *Clostridium butyricum*, *Clostridium sp.*, *Enterococcus faecium*, *Enterococcus sp.*, *Lactobacillus sp.*, *Lactobacillus acidophilus*, *Lactobacillus farciminus*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactococcus lactis*, *Lactococcus sp.*, *Leuconostoc sp.*,
20 *Megasphaera elsdenii*, *Megasphaera sp.*, *Pediococcus acidilactici*, *Pediococcus sp.*, *Propionibacterium thoenii*, *Propionibacterium sp.* and *Streptococcus sp.* or any combination thereof.

25

Sources of Polypeptides Having alpha-galactosidase Activity

A polypeptide having alpha-galactosidase activity of the present invention may be obtained from microorganisms of any genus. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide
30 encoded by a polynucleotide is produced by the source or by a strain in which the polynucleotide from the source has been inserted. In one aspect, the polypeptide obtained from a given source is secreted extracellularly.

In one embodiment, the polypeptide is from a fungus of the class *Eurotiomycetes*, such as from the order *Eurotiales*, or from the family *Aspergillaceae*, or from the genus *Penicillium* or
35 from the species *Penicillium pseudopulvillorum*.

It will be understood that for the aforementioned species, the invention encompasses both the perfect and imperfect states, and other taxonomic equivalents, e.g., anamorphs, regardless

of the species name by which they are known. Those skilled in the art will readily recognize the identity of appropriate equivalents.

5 Strains of these species are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

10 The polypeptide may be identified and obtained from other sources including microorganisms isolated from nature (*e.g.*, soil, composts, water, etc.) or DNA samples obtained directly from natural materials (*e.g.*, soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms and DNA directly from natural habitats are well known in the art. A polynucleotide encoding the polypeptide may then be obtained by similarly screening a genomic DNA or cDNA library of another microorganism or mixed DNA sample. Once a polynucleotide encoding a polypeptide has been detected with the probe(s), the polynucleotide
15 can be isolated or cloned by utilizing techniques that are known to those of ordinary skill in the art (see, *e.g.*, Sambrook *et al.*, 1989, *supra*).

Nucleic Acid Constructs

20 The present invention also relates to nucleic acid constructs comprising a polynucleotide of the present invention operably linked to one or more control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

The polynucleotide may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying
25 polynucleotides utilizing recombinant DNA methods are well known in the art.

The control sequence may be a promoter, a polynucleotide that is recognized by a host cell for expression of a polynucleotide encoding a polypeptide of the present invention. The promoter contains transcriptional control sequences that mediate the expression of the polypeptide. The promoter may be any polynucleotide that shows transcriptional activity in the
30 host cell including variant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a bacterial host cell are the promoters obtained from the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus subtilis* *xylA* and *xylB*

genes, *Bacillus thuringiensis cryIIIA* gene (Agaisse and Lereclus, 1994, *Molecular Microbiology* 13: 97-107), *E. coli lac* operon, *E. coli trc* promoter (Egon *et al.*, 1988, *Gene* 69: 301-315), *Streptomyces coelicolor* agarase gene (*dagA*), and prokaryotic beta-lactamase gene (Villa-Kamaroff *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 3727-3731), as well as the *tac* promoter
5 (DeBoer *et al.*, 1983, *Proc. Natl. Acad. Sci. USA* 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Gilbert *et al.*, 1980, *Scientific American* 242: 74-94; and in Sambrook *et al.*, 1989, *supra*. Examples of tandem promoters are disclosed in WO 99/43835.

Examples of suitable promoters for directing transcription of the nucleic acid constructs of
10 the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Aspergillus oryzae* TAKA amylase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Fusarium venenatum*
15 amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Rhizomucor miehei* lipase, *Rhizomucor miehei* aspartic proteinase, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei*
20 endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase III, *Trichoderma reesei* beta-xylosidase, and *Trichoderma reesei* translation elongation factor, as well as the NA2-tpi promoter (a modified promoter from an *Aspergillus* neutral alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus* triose phosphate isomerase gene; non-limiting examples
25 include modified promoters from an *Aspergillus niger* neutral alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus nidulans* or *Aspergillus oryzae* triose phosphate isomerase gene); and variant, truncated, and hybrid promoters thereof. Other promoters are described in U.S. Patent No. 6,011,147.

In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae*
30 *cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH1, ADH2/GAP), *Saccharomyces cerevisiae* triose phosphate isomerase (TPI), *Saccharomyces cerevisiae* metallothionein (CUP1), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are described by Romanos *et al.*, 1992, *Yeast* 8: 423-
35 488.

The control sequence may also be a transcription terminator, which is recognized by a host cell to terminate transcription. The terminator is operably linked to the 3'-terminus of the

polynucleotide encoding the polypeptide. Any terminator that is functional in the host cell may be used in the present invention.

Preferred terminators for bacterial host cells are obtained from the genes for *Bacillus clausii* alkaline protease (*aprH*), *Bacillus licheniformis* alpha-amylase (*amyL*), and *Escherichia coli* ribosomal RNA (*rrnB*).

Preferred terminators for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, *Fusarium oxysporum* trypsin-like protease, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase III, *Trichoderma reesei* beta-xylosidase, and *Trichoderma reesei* translation elongation factor.

Preferred terminators for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be an mRNA stabilizer region downstream of a promoter and upstream of the coding sequence of a gene which increases expression of the gene.

Examples of suitable mRNA stabilizer regions are obtained from a *Bacillus thuringiensis cryIIIA* gene (WO 94/25612) and a *Bacillus subtilis* SP82 gene (Hue *et al.*, 1995, *Journal of Bacteriology* 177: 3465-3471).

The control sequence may also be a leader, a nontranslated region of an mRNA that is important for translation by the host cell. The leader is operably linked to the 5'-terminus of the polynucleotide encoding the polypeptide. Any leader that is functional in the host cell may be used.

Preferred leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase and *Aspergillus nidulans* triose phosphate isomerase.

Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3'-terminus of the polynucleotide and, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence that is functional in the host cell may be used.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

5 Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, *Mol. Cellular Biol.* 15: 5983-5990.

The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a polypeptide and directs the polypeptide into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently
10 contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the polypeptide. Alternatively, the 5'-end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. A foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, a foreign signal peptide
15 coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the polypeptide. However, any signal peptide coding sequence that directs the expressed polypeptide into the secretory pathway of a host cell may be used.

Effective signal peptide coding sequences for bacterial host cells are the signal peptide coding sequences obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus*
20 *licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus stearothermophilus* neutral proteases (*nprT*, *nprS*, *nprM*), and *Bacillus subtilis* *prsA*. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

Effective signal peptide coding sequences for filamentous fungal host cells are the signal
25 peptide coding sequences obtained from the genes for *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Aspergillus oryzae* TAKA amylase, *Humicola insolens* cellulase, *Humicola insolens* endoglucanase V, *Humicola lanuginosa* lipase, and *Rhizomucor miehei* aspartic proteinase.

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces*
30 *cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding sequences are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be a propeptide coding sequence that encodes a propeptide positioned at the N-terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally
35 inactive and can be converted to an active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding sequence may be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*), *Bacillus subtilis* neutral protease (*nprT*),

Myceliophthora thermophila laccase (WO 95/33836), *Rhizomucor miehei* aspartic proteinase, and *Saccharomyces cerevisiae* alpha-factor.

5 Where both signal peptide and propeptide sequences are present, the propeptide sequence is positioned next to the N-terminus of a polypeptide and the signal peptide sequence is positioned next to the N-terminus of the propeptide sequence.

10 It may also be desirable to add regulatory sequences that regulate expression of the polypeptide relative to the growth of the host cell. Examples of regulatory sequences are those that cause expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory sequences in prokaryotic systems include the *lac*, *tac*, and *trp* operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the *Aspergillus niger* glucoamylase promoter, *Aspergillus oryzae* TAKA alpha-amylase promoter, and *Aspergillus oryzae* glucoamylase promoter, *Trichoderma reesei* cellobiohydrolase I promoter, and *Trichoderma reesei* cellobiohydrolase II promoter may be used. Other examples of regulatory sequences are those
15 that allow for gene amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the polynucleotide encoding the polypeptide would be operably linked to the regulatory sequence.

Expression Vectors

20 The present invention also relates to recombinant expression vectors comprising a polynucleotide of the present invention, a promoter, and transcriptional and translational stop signals. The various nucleotide and control sequences may be joined together to produce a recombinant expression vector that may include one or more convenient restriction sites to allow for insertion or substitution of the polynucleotide encoding the polypeptide at such sites.
25 Alternatively, the polynucleotide may be expressed by inserting the polynucleotide or a nucleic acid construct comprising the polynucleotide into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

30 The recombinant expression vector may be any vector (*e.g.*, a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the polynucleotide. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vector may be a linear or closed circular plasmid.

35 The vector may be an autonomously replicating vector, *i.e.*, a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one

that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

5 The vector preferably contains one or more selectable markers that permit easy selection of transformed, transfected, transduced, or the like cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

10 Examples of bacterial selectable markers are *Bacillus licheniformis* or *Bacillus subtilis* *dal* genes, or markers that confer antibiotic resistance such as ampicillin, chloramphenicol, kanamycin, neomycin, spectinomycin, or tetracycline resistance. Suitable markers for yeast host cells include, but are not limited to, ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in a filamentous fungal host cell include, but are not limited to, *adeA* (phosphoribosylaminoimidazole-succinocarboxamide synthase), *adeB* (phosphoribosyl-
15 aminoimidazole synthase), *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are *Aspergillus nidulans* or *Aspergillus oryzae* *amdS* and *pyrG* genes and a *Streptomyces*
20 *hygroscopicus* *bar* gene. Preferred for use in a *Trichoderma* cell are *adeA*, *adeB*, *amdS*, *hph*, and *pyrG* genes.

The selectable marker may be a dual selectable marker system as described in WO 2010/039889. In one aspect, the dual selectable marker is an *hph-tk* dual selectable marker system.

25 The vector preferably contains an element(s) that permits integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.

For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the polypeptide or any other element of the vector for integration into the genome by homologous or non-homologous recombination. Alternatively, the vector may contain
30 additional polynucleotides for directing integration by homologous recombination into the genome of the host cell at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, 400 to 10,000 base pairs, and 800 to 10,000 base pairs, which have a high degree of sequence identity to the corresponding target sequence
35 to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding polynucleotides. On

the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator" means a polynucleotide that enables a plasmid or vector to replicate *in vivo*.

Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, and pAM β 1 permitting replication in *Bacillus*.

Examples of origins of replication for use in a yeast host cell are the 2 micron origin of replication, ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6.

Examples of origins of replication useful in a filamentous fungal cell are AMA1 and ANS1 (Gems *et al.*, 1991, *Gene* 98: 61-67; Cullen *et al.*, 1987, *Nucleic Acids Res.* 15: 9163-9175; WO 00/24883). Isolation of the AMA1 gene and construction of plasmids or vectors comprising the gene can be accomplished according to the methods disclosed in WO 00/24883.

More than one copy of a polynucleotide of the present invention may be inserted into a host cell to increase production of a polypeptide. An increase in the copy number of the polynucleotide can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the polynucleotide where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the polynucleotide, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, *e.g.*, Sambrook *et al.*, 1989, *supra*).

Host Cells

The present invention also relates to recombinant host cells, comprising a polynucleotide of the present invention operably linked to one or more control sequences that direct the production of a polypeptide of the present invention. A construct or vector comprising a polynucleotide is introduced into a host cell so that the construct or vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source.

The host cell may be any cell useful in the recombinant production of a polypeptide of the present invention, e.g., a prokaryote or a eukaryote.

The prokaryotic host cell may be any Gram-positive or Gram-negative bacterium. Gram-positive bacteria include, but are not limited to, *Bacillus*, *Clostridium*, *Enterococcus*, *Geobacillus*,
5 *Lactobacillus*, *Lactococcus*, *Oceanobacillus*, *Staphylococcus*, *Streptococcus*, and *Streptomyces*. Gram-negative bacteria include, but are not limited to, *Campylobacter*, *E. coli*, *Flavobacterium*, *Fusobacterium*, *Helicobacter*, *Ilyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*, and *Ureaplasma*.

The bacterial host cell may be any *Bacillus* cell including, but not limited to, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*,
10 *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis* cells.

The bacterial host cell may also be any *Streptococcus* cell including, but not limited to, *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, and *Streptococcus equi* subsp. *Zooepidemicus* cells.
15

The bacterial host cell may also be any *Streptomyces* cell including, but not limited to, *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, and *Streptomyces lividans* cells.

The introduction of DNA into a *Bacillus* cell may be effected by protoplast transformation
20 (see, e.g., Chang and Cohen, 1979, *Mol. Gen. Genet.* 168: 111-115), competent cell transformation (see, e.g., Young and Spizizen, 1961, *J. Bacteriol.* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *J. Mol. Biol.* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *J. Bacteriol.* 169: 5271-5278). The introduction of DNA into an *E. coli* cell may be effected by
25 protoplast transformation (see, e.g., Hanahan, 1983, *J. Mol. Biol.* 166: 557-580) or electroporation (see, e.g., Dower *et al.*, 1988, *Nucleic Acids Res.* 16: 6127-6145). The introduction of DNA into a *Streptomyces* cell may be effected by protoplast transformation, electroporation (see, e.g., Gong *et al.*, 2004, *Folia Microbiol. (Praha)* 49: 399-405), conjugation (see, e.g., Mazodier *et al.*, 1989, *J. Bacteriol.* 171: 3583-3585), or transduction (see, e.g., Burke *et al.*, 2001, *Proc. Natl. Acad. Sci.*
30 *USA* 98: 6289-6294). The introduction of DNA into a *Pseudomonas* cell may be effected by electroporation (see, e.g., Choi *et al.*, 2006, *J. Microbiol. Methods* 64: 391-397) or conjugation (see, e.g., Pinedo and Smets, 2005, *Appl. Environ. Microbiol.* 71: 51-57). The introduction of DNA into a *Streptococcus* cell may be effected by natural competence (see, e.g., Perry and Kuramitsu, 1981, *Infect. Immun.* 32: 1295-1297), protoplast transformation (see, e.g., Catt and Jollick, 1991,
35 *Microbios* 68: 189-207), electroporation (see, e.g., Buckley *et al.*, 1999, *Appl. Environ. Microbiol.* 65: 3800-3804), or conjugation (see, e.g., Clewell, 1981, *Microbiol. Rev.* 45: 409-436). However, any method known in the art for introducing DNA into a host cell can be used.

The host cell may also be a eukaryote, such as a mammalian, insect, plant, or fungal cell.

The host cell may be a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota as well as the Oomycota and all mitosporic fungi (as defined by Hawksworth *et al.*, In, *Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK).

The fungal host cell may be a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (*Endomycetales*), basidiosporogenous yeast, and yeast belonging to the *Fungi Imperfecti* (*Blastomycetes*). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, Passmore, and Davenport, editors, *Soc. App. Bacteriol. Symposium Series No. 9*, 1980).

The yeast host cell may be a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell, such as a *Kluyveromyces lactis*, *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, *Saccharomyces oviformis*, or *Yarrowia lipolytica* cell.

The fungal host cell may be a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

The filamentous fungal host cell may be an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma* cell.

For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*,

Fusarium negundi, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP 238023, Yelton *et al.*, 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474, and Christensen *et al.*, 1988, *Bio/Technology* 6: 1419-1422. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156, and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *J. Bacteriol.* 153: 163; and Hinnen *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 1920.

Methods of Production

The present invention also relates to methods of producing a polypeptide of the present invention, comprising (a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and optionally, (b) recovering the polypeptide. In one aspect, the cell is a *Penicillium* cell. In another aspect, the cell is a *Penicillium pseudopulvillorum* cell.

The present invention also relates to methods of producing a polypeptide of the present invention, comprising (a) cultivating a recombinant host cell of the present invention under conditions conducive for production of the polypeptide; and optionally, (b) recovering the polypeptide.

The host cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cells may be cultivated by shake flask cultivation, or small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be

recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The polypeptide may be detected using methods known in the art that are specific for the polypeptides alpha-galactosidase activity. These detection methods include, but are not limited to, use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the polypeptide.

The polypeptide may be recovered using methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, collection, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. In one aspect, a fermentation broth comprising the polypeptide is recovered.

The polypeptide may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., *Protein Purification*, Janson and Ryden, editors, VCH Publishers, New York, 1989) to obtain substantially pure polypeptides.

In an alternative aspect, the polypeptide is not recovered, but rather a host cell of the present invention expressing the polypeptide is used as a source of the polypeptide.

20 **Production in plants**

The present invention also relates to isolated plants, e.g., a transgenic plant, plant part, or plant cell, comprising a polynucleotide of the present invention so as to express and produce a polypeptide or domain in recoverable quantities. The polypeptide or domain may be recovered from the plant or plant part. Alternatively, the plant or plant part containing the polypeptide or domain may be used as such for improving the quality of a food or feed, e.g., improving nutritional value, palatability, and rheological properties, or to destroy an antinutritive factor.

The transgenic plant can be dicotyledonous (a dicot) or monocotyledonous (a monocot). Examples of monocot plants are grasses, such as meadow grass (blue grass, *Poa*), forage grass such as *Festuca*, *Lolium*, temperate grass, such as *Agrostis*, and cereals, e.g., wheat, oats, rye, barley, rice, sorghum, and maize (corn).

Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous plants (family *Brassicaceae*), such as cauliflower, rape seed, and the closely related model organism *Arabidopsis thaliana*.

Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers as well as the individual tissues comprising these parts, e.g., epidermis, mesophyll, parenchyme, vascular tissues, meristems.

Plant cells and specific plant cell compartments, such as chloroplasts, apoplasts, mitochondria, vacuoles, peroxisomes and cytoplasm are also considered to be a plant part.

Also included within the scope of the present invention are the progeny of such plants, plant parts, and plant cells.

5 The transgenic plant or plant cell expressing the polypeptide or domain may be constructed in accordance with methods known in the art.

The present invention also relates to methods of producing a polypeptide or domain of the present invention comprising (a) cultivating a transgenic plant or a plant cell comprising a polynucleotide encoding the polypeptide or domain under conditions conducive for production of
10 the polypeptide or domain; and (b) recovering the polypeptide or domain.

Fermentation Broth Formulations or Cell Compositions

The present invention also relates to a fermentation broth formulation or a cell composition comprising a polypeptide of the present invention. The fermentation broth product further comprises additional ingredients used in the fermentation process, such as, for example, cells
15 (including, the host cells containing the gene encoding the polypeptide of the present invention which are used to produce the polypeptide of interest), cell debris, biomass, fermentation media and/or fermentation products. In some embodiments, the composition is a cell-killed whole broth containing organic acid(s), killed cells and/or cell debris, and culture medium.

The term "fermentation broth" as used herein refers to a preparation produced by cellular
20 fermentation that undergoes no or minimal recovery and/or purification. For example, fermentation broths are produced when microbial cultures are grown to saturation, incubated under carbon-limiting conditions to allow protein synthesis (*e.g.*, expression of enzymes by host cells) and secretion into cell culture medium. The fermentation broth can contain unfractionated or fractionated contents of the fermentation materials derived at the end of the fermentation.
25 Typically, the fermentation broth is unfractionated and comprises the spent culture medium and cell debris present after the microbial cells (*e.g.*, filamentous fungal cells) are removed, *e.g.*, by centrifugation. In some embodiments, the fermentation broth contains spent cell culture medium, extracellular enzymes, and viable and/or nonviable microbial cells.

In an embodiment, the fermentation broth formulation and cell compositions comprise a
30 first organic acid component comprising at least one 1-5 carbon organic acid and/or a salt thereof and a second organic acid component comprising at least one 6 or more carbon organic acid and/or a salt thereof. In a specific embodiment, the first organic acid component is acetic acid, formic acid, propionic acid, a salt thereof, or a mixture of two or more of the foregoing and the second organic acid component is benzoic acid, cyclohexanecarboxylic acid, 4-methylvaleric
35 acid, phenylacetic acid, a salt thereof, or a mixture of two or more of the foregoing.

In one aspect, the composition contains an organic acid(s), and optionally further contains killed cells and/or cell debris. In one embodiment, the killed cells and/or cell debris are removed from a cell-killed whole broth to provide a composition that is free of these components.

5 The fermentation broth formulations or cell compositions may further comprise a preservative and/or anti-microbial (e.g., bacteriostatic) agent, including, but not limited to, sorbitol, sodium chloride, potassium sorbate, and others known in the art.

10 The cell-killed whole broth or composition may contain the unfractionated contents of the fermentation materials derived at the end of the fermentation. Typically, the cell-killed whole broth or composition contains the spent culture medium and cell debris present after the microbial cells (e.g., filamentous fungal cells) are grown to saturation, incubated under carbon-limiting conditions to allow protein synthesis. In some embodiments, the cell-killed whole broth or composition contains the spent cell culture medium, extracellular enzymes, and killed filamentous fungal cells. In some embodiments, the microbial cells present in the cell-killed whole broth or composition can be permeabilized and/or lysed using methods known in the art.

15 A whole broth or cell composition as described herein is typically a liquid, but may contain insoluble components, such as killed cells, cell debris, culture media components, and/or insoluble enzyme(s). In some embodiments, insoluble components may be removed to provide a clarified liquid composition.

20 The whole broth formulations and cell compositions of the present invention may be produced by a method described in WO 90/15861 or WO 2010/096673.

Enzyme Compositions

The present invention also relates to compositions comprising a polypeptide of the present invention. Preferably, the compositions are enriched in the polypeptide of the invention. The term "enriched" indicates that the alpha-galactosidase activity of the composition has been increased, e.g., with an enrichment factor of at least 1.1, such as at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 2.0, at least 3.0, at least 4.0, at least 5.0, at least 10.

In an embodiment, the composition comprises one or more polypeptides of the second aspect of the invention and one or more formulating agents, as described below.

30 The compositions may further comprise multiple enzymatic activities, such as one or more (e.g., several) enzymes selected from the group consisting of acetylxylan esterase, acylglycerol lipase, amylase, alpha-amylase, beta-amylase, arabinofuranosidase, cellobiohydrolases, cellulase, feruloyl esterase, galactanase, alpha-galactosidase, beta-galactosidase, beta-glucanase, beta-glucosidase, lysophospholipase, lysozyme, alpha-mannosidase, beta-mannosidase (mannanase), phytase, phospholipase A1, phospholipase A2, phospholipase D, 35 protease, pullulanase, pectinesterase, triacylglycerol lipase, xylanase, beta-xylosidase or any combination thereof.

The compositions may further comprise one or more microbes. In an embodiment, the microbe is selected from the group consisting of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus pumilus*, *Bacillus polymyxa*, *Bacillus megaterium*, *Bacillus coagulans*, *Bacillus circulans*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*,
5 *Bifidobacterium sp.*, *Carnobacterium sp.*, *Clostridium butyricum*, *Clostridium sp.*, *Enterococcus faecium*, *Enterococcus sp.*, *Lactobacillus sp.*, *Lactobacillus acidophilus*, *Lactobacillus farciminus*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactococcus lactis*, *Lactococcus sp.*, *Leuconostoc sp.*, *Megasphaera elsdenii*, *Megasphaera sp.*, *Pediococcus acidilactici*, *Pediococcus sp.*, *Propionibacterium thoenii*, *Propionibacterium sp.* and *Streptococcus*
10 *sp.* or any combination thereof.

Formulation

The enzyme of the invention may be formulated as a liquid or a solid. For a liquid formulation, the formulating agent may comprise a polyol (such as e.g. glycerol, ethylene glycol or propylene glycol), a salt (such as e.g. sodium chloride, sodium benzoate, potassium sorbate)
15 or a sugar or sugar derivative (such as e.g. dextrin, glucose, sucrose, and sorbitol). Thus in one embodiment, the composition is a liquid composition comprising the polypeptide of the invention and one or more formulating agents selected from the list consisting of glycerol, ethylene glycol, 1,2-propylene glycol, 1,3-propylene glycol, sodium chloride, sodium benzoate, potassium sorbate, dextrin, glucose, sucrose, and sorbitol. The liquid formulation may be sprayed onto the
20 feed after it has been pelleted or may be added to drinking water given to the animals.

For a solid formulation, the formulation may be for example as a granule, spray dried powder or agglomerate (e.g. as disclosed in WO2000/70034). The formulating agent may comprise a salt (organic or inorganic zinc, sodium, potassium or calcium salts such as e.g. such as calcium acetate, calcium benzoate, calcium carbonate, calcium chloride, calcium citrate,
25 calcium sorbate, calcium sulfate, potassium acetate, potassium benzoate, potassium carbonate, potassium chloride, potassium citrate, potassium sorbate, potassium sulfate, sodium acetate, sodium benzoate, sodium carbonate, sodium chloride, sodium citrate, sodium sulfate, zinc acetate, zinc benzoate, zinc carbonate, zinc chloride, zinc citrate, zinc sorbate, zinc sulfate), starch or a sugar or sugar derivative (such as e.g. sucrose, dextrin, glucose, lactose, sorbitol).

30 In one embodiment, the composition is a solid composition, such as a spray dried composition, comprising the alpha-galactosidase of the invention and one or more formulating agents selected from the list consisting of sodium chloride, sodium benzoate, potassium sorbate, sodium sulfate, potassium sulfate, magnesium sulfate, sodium thiosulfate, calcium carbonate, sodium citrate, dextrin, glucose, sucrose, sorbitol, lactose, starch and cellulose. In a preferred
35 embodiment, the formulating agent is selected from one or more of the following compounds: sodium sulfate, dextrin, cellulose, sodium thiosulfate, magnesium sulfate and calcium carbonate.

The present invention also relates to enzyme granules/particles comprising the alpha-galactosidase of the invention optionally combined with one or more additional enzymes. The granule is composed of a core, and optionally one or more coatings (outer layers) surrounding the core.

5 Typically the granule/particle size, measured as equivalent spherical diameter (volume based average particle size), of the granule is 20-2000 μm , particularly 50-1500 μm , 100-1500 μm or 250-1200 μm .

The core can be prepared by granulating a blend of the ingredients, e.g., by a method comprising granulation techniques such as crystallization, precipitation, pan-coating, fluid bed
10 coating, fluid bed agglomeration, rotary atomization, extrusion, prilling, spheronization, size reduction methods, drum granulation, and/or high shear granulation.

Methods for preparing the core can be found in Handbook of Powder Technology; Particle size enlargement by C. E. Capes; Volume 1; 1980; Elsevier. Preparation methods include known feed and granule formulation technologies, e.g.:

15 a) spray dried products, wherein a liquid enzyme-containing solution is atomized in a spray drying tower to form small droplets which during their way down the drying tower dry to form an enzyme-containing particulate material;

b) layered products, wherein the enzyme is coated as a layer around a pre-formed inert core particle, wherein an enzyme-containing solution is atomized, typically in a fluid bed apparatus
20 wherein the pre-formed core particles are fluidized, and the enzyme-containing solution adheres to the core particles and dries up to leave a layer of dry enzyme on the surface of the core particle. Particles of a desired size can be obtained this way if a useful core particle of the desired size can be found. This type of product is described in, e.g., WO 97/23606;

c) absorbed core particles, wherein rather than coating the enzyme as a layer around the
25 core, the enzyme is absorbed onto and/or into the surface of the core. Such a process is described in WO 97/39116.

d) extrusion or pelletized products, wherein an enzyme-containing paste is pressed to pellets or under pressure is extruded through a small opening and cut into particles which are
30 subsequently dried. Such particles usually have a considerable size because of the material in which the extrusion opening is made (usually a plate with bore holes) sets a limit on the allowable pressure drop over the extrusion opening. Also, very high extrusion pressures when using a small opening increase heat generation in the enzyme paste, which is harmful to the enzyme;

e) prilled products, wherein an enzyme-containing powder is suspended in molten wax and the suspension is sprayed, e.g., through a rotating disk atomiser, into a cooling chamber
35 where the droplets quickly solidify (Michael S. Showell (editor); *Powdered detergents*; Surfactant Science Series; 1998; vol. 71; page 140-142; Marcel Dekker). The product obtained is one wherein the enzyme is uniformly distributed throughout an inert material instead of being

concentrated on its surface. Also US 4,016,040 and US 4,713,245 are documents relating to this technique;

f) mixer granulation products, wherein a liquid is added to a dry powder composition of, e.g., conventional granulating components, the enzyme being introduced either via the liquid or the powder or both. The liquid and the powder are mixed and as the moisture of the liquid is absorbed in the dry powder, the components of the dry powder will start to adhere and agglomerate and particles will build up, forming granulates comprising the enzyme. Such a process is described in US 4,106,991 and related documents EP 170360, EP 304332, EP 304331, WO 90/09440 and WO 90/09428. In a particular product of this process wherein various high-shear mixers can be used as granulators, granulates consisting of enzyme as enzyme, fillers and binders etc. are mixed with cellulose fibres to reinforce the particles to give the so-called T-granulate. Reinforced particles, being more robust, release less enzymatic dust.

g) size reduction, wherein the cores are produced by milling or crushing of larger particles, pellets, tablets, briquettes etc. containing the enzyme. The wanted core particle fraction is obtained by sieving the milled or crushed product. Over and undersized particles can be recycled. Size reduction is described in (Martin Rhodes (editor); Principles of Powder Technology; 1990; Chapter 10; John Wiley & Sons);

h) fluid bed granulation, which involves suspending particulates in an air stream and spraying a liquid onto the fluidized particles via nozzles. Particles hit by spray droplets get wetted and become tacky. The tacky particles collide with other particles and adhere to them and form a granule;

i) the cores may be subjected to drying, such as in a fluid bed drier. Other known methods for drying granules in the feed or detergent industry can be used by the skilled person. The drying preferably takes place at a product temperature of from 25 to 90°C. For some enzymes it is important the cores comprising the enzyme contain a low amount of water before coating. If water sensitive enzymes are coated before excessive water is removed, it will be trapped within the core and it may affect the activity of the enzyme negatively. After drying, the cores preferably contain 0.1-10 % w/w water.

The core may include additional materials such as fillers, fibre materials (cellulose or synthetic fibres), stabilizing agents, solubilizing agents, suspension agents, viscosity regulating agents, light spheres, plasticizers, salts, lubricants and fragrances.

The core may include a binder, such as synthetic polymer, wax, fat, or carbohydrate.

The core may include a salt of a multivalent cation, a reducing agent, an antioxidant, a peroxide decomposing catalyst and/or an acidic buffer component, typically as a homogenous blend.

In one embodiment, the core comprises a material selected from the group consisting of salts (such as calcium acetate, calcium benzoate, calcium carbonate, calcium chloride, calcium

citrate, calcium sorbate, calcium sulfate, potassium acetate, potassium benzoate, potassium carbonate, potassium chloride, potassium citrate, potassium sorbate, potassium sulfate, sodium acetate, sodium benzoate, sodium carbonate, sodium chloride, sodium citrate, sodium sulfate, zinc acetate, zinc benzoate, zinc carbonate, zinc chloride, zinc citrate, zinc sorbate, zinc sulfate),
5 starch or a sugar or sugar derivative (such as e.g. sucrose, dextrin, glucose, lactose, sorbitol), sugar or sugar derivative (such as e.g. sucrose, dextrin, glucose, lactose, sorbitol), small organic molecules, starch, flour, cellulose and minerals and clay minerals (also known as hydrous aluminium phyllosilicates). In one embodiment, the core comprises a clay mineral such as kaolinite or kaolin.

10 The core may include an inert particle with the enzyme absorbed into it, or applied onto the surface, e.g., by fluid bed coating.

The core may have a diameter of 20-2000 μm , particularly 50-1500 μm , 100-1500 μm or 250-1200 μm .

15 The core may be surrounded by at least one coating, e.g., to improve the storage stability, to reduce dust formation during handling, or for coloring the granule. The optional coating(s) may include a salt and/or wax and/or flour coating, or other suitable coating materials.

The coating may be applied in an amount of at least 0.1% by weight of the core, e.g., at least 0.5%, 1% or 5%. The amount may be at most 100%, 70%, 50%, 40% or 30%.

20 The coating is preferably at least 0.1 μm thick, particularly at least 0.5 μm , at least 1 μm or at least 5 μm . In some embodiments the thickness of the coating is below 100 μm , such as below 60 μm , or below 40 μm .

25 The coating should encapsulate the core unit by forming a substantially continuous layer. A substantially continuous layer is to be understood as a coating having few or no holes, so that the core unit is encapsulated or enclosed with few or no uncoated areas. The layer or coating should in particular be homogeneous in thickness.

The coating can further contain other materials as known in the art, e.g., fillers, antisticking agents, pigments, dyes, plasticizers and/or binders, such as titanium dioxide, kaolin, calcium carbonate or talc.

30 A salt coating may comprise at least 60% by weight of a salt, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99% by weight.

The salt may be added from a salt solution where the salt is completely dissolved or from a salt suspension wherein the fine particles are less than 50 μm , such as less than 10 μm or less than 5 μm .

35 The salt coating may comprise a single salt or a mixture of two or more salts. The salt may be water soluble, in particular having a solubility at least 0.1 g in 100 g of water at 20°C, preferably at least 0.5 g per 100 g water, e.g., at least 1 g per 100 g water, e.g., at least 5 g per 100 g water.

The salt may be an inorganic salt, e.g., salts of sulfate, sulfite, phosphate, phosphonate, nitrate, chloride or carbonate or salts of simple organic acids (less than 10 carbon atoms, e.g., 6 or less carbon atoms) such as citrate, malonate or acetate. Examples of cations in these salts are alkali or earth alkali metal ions, the ammonium ion or metal ions of the first transition series, such as sodium, potassium, magnesium, calcium, zinc or aluminium. Examples of anions include chloride, bromide, iodide, sulfate, sulfite, bisulfite, thiosulfate, phosphate, monobasic phosphate, dibasic phosphate, hypophosphite, dihydrogen pyrophosphate, tetraborate, borate, carbonate, bicarbonate, metasilicate, citrate, malate, maleate, malonate, succinate, sorbate, lactate, formate, acetate, butyrate, propionate, benzoate, tartrate, ascorbate or gluconate. In particular alkali- or earth alkali metal salts of sulfate, sulfite, phosphate, phosphonate, nitrate, chloride or carbonate or salts of simple organic acids such as citrate, malonate or acetate may be used.

The salt in the coating may have a constant humidity at 20°C above 60%, particularly above 70%, above 80% or above 85%, or it may be another hydrate form of such a salt (e.g., anhydrate). The salt coating may be as described in WO1997/05245, WO1998/54980, WO1998/55599, WO2000/70034, WO2006/034710, WO2008/017661, WO2008/017659, WO2000/020569, WO2001/004279, WO1997/05245, WO2000/01793, WO2003/059086, WO2003/059087, WO2007/031483, WO2007/031485, WO2007/044968, WO2013/192043, WO2014/014647 and WO2015/197719 or polymer coating such as described in WO 2001/00042.

Specific examples of suitable salts are NaCl (CH₂₀°C=76%), Na₂CO₃ (CH₂₀°C=92%), NaNO₃ (CH₂₀°C=73%), Na₂HPO₄ (CH₂₀°C=95%), Na₃PO₄ (CH₂₅°C=92%), NH₄Cl (CH₂₀°C = 79.5%), (NH₄)₂HPO₄ (CH₂₀°C = 93,0%), NH₄H₂PO₄ (CH₂₀°C = 93.1%), (NH₄)₂SO₄ (CH₂₀°C=81.1%), KCl (CH₂₀°C=85%), K₂HPO₄ (CH₂₀°C=92%), KH₂PO₄ (CH₂₀°C=96.5%), KNO₃ (CH₂₀°C=93.5%), Na₂SO₄ (CH₂₀°C=93%), K₂SO₄ (CH₂₀°C=98%), KHSO₄ (CH₂₀°C=86%), MgSO₄ (CH₂₀°C=90%), ZnSO₄ (CH₂₀°C=90%) and sodium citrate (CH₂₅°C=86%). Other examples include NaH₂PO₄, (NH₄)H₂PO₄, CuSO₄, Mg(NO₃)₂, magnesium acetate, calcium acetate, calcium benzoate, calcium carbonate, calcium chloride, calcium citrate, calcium sorbate, calcium sulfate, potassium acetate, potassium benzoate, potassium carbonate, potassium chloride, potassium citrate, potassium sorbate, sodium acetate, sodium benzoate, sodium citrate, sodium sulfate, zinc acetate, zinc benzoate, zinc carbonate, zinc chloride, zinc citrate and zinc sorbate.

The salt may be in anhydrous form, or it may be a hydrated salt, i.e. a crystalline salt hydrate with bound water(s) of crystallization, such as described in WO 99/32595. Specific examples include anhydrous sodium sulfate (Na₂SO₄), anhydrous magnesium sulfate (MgSO₄), magnesium sulfate heptahydrate (MgSO₄.7H₂O), zinc sulfate heptahydrate (ZnSO₄.7H₂O), sodium phosphate dibasic heptahydrate (Na₂HPO₄.7H₂O), magnesium nitrate hexahydrate (Mg(NO₃)₂(6H₂O)), sodium citrate dihydrate and magnesium acetate tetrahydrate.

Preferably the salt is applied as a solution of the salt, e.g., using a fluid bed.

A wax coating may comprise at least 60% by weight of a wax, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99% by weight.

Specific examples of waxes are polyethylene glycols; polypropylenes; Carnauba wax; Candelilla wax; bees wax; hydrogenated plant oil or animal tallow such as polyethylene glycol (PEG), methyl hydroxy-propyl cellulose (MHPC), polyvinyl alcohol (PVA), hydrogenated ox tallow, hydrogenated palm oil, hydrogenated cotton seeds and/or hydrogenated soy bean oil; fatty acid alcohols; mono-glycerides and/or di-glycerides, such as glyceryl stearate, wherein stearate is a mixture of stearic and palmitic acid; micro-crystalline wax; paraffin's; and fatty acids, such as hydrogenated linear long chained fatty acids and derivatives thereof. A preferred wax is palm oil or hydrogenated palm oil.

The granule may comprise a core comprising the alpha-galactosidase of the invention, one or more salt coatings and one or more wax coatings. Examples of enzyme granules with multiple coatings are shown in WO1993/07263, WO1997/23606 and WO2016/149636.

Non-dusting granulates may be produced, e.g., as disclosed in U.S. Patent Nos. 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. The coating materials can be waxy coating materials and film-forming coating materials. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591.

The granulate may further comprise one or more additional enzymes. Each enzyme will then be present in more granules securing a more uniform distribution of the enzymes, and also reduces the physical segregation of different enzymes due to different particle sizes. Methods for producing multi-enzyme co-granulates is disclosed in the ip.com disclosure IPCOM000200739D.

Another example of formulation of enzymes by the use of co-granulates is disclosed in WO 2013/188331.

The present invention also relates to protected enzymes prepared according to the method disclosed in EP 238,216.

Thus, in a further aspect, the present invention provides a granule, which comprises:

- (a) a core comprising an alpha-galactosidase according to the invention, and
- (b) a coating consisting of one or more layer(s) surrounding the core.

In one embodiment, the coating comprises a salt coating as described herein. In one embodiment, the coating comprises a wax coating as described herein. In one embodiment, the coating comprises a salt coating followed by a wax coating as described herein.

Plant based material

In an embodiment, the plant based material is from the taxonomic subclass *rosids* such as the taxonomic order *Fabales* or the the taxonomic order *Brassicales*.

In one embodiment, the plant based material from is from the family *Fabaceae*, such as
 5 the subfamilies *Caesalpinioideae* or *Mimosoideae* or *Papilionoideae*. In an embodiment, the plant based material from is from the sub-family *Papilionoideae*, such as the tribe *Abreae* or *Amorpheae* or *Bossiaeeae* or *Brongniartieae* or *Cicereae* or *Crotalariaeae* or *Dalbergieae* or *Desmodieae* or *Dipterygeae* or *Euchresteeae* or *Fabeae* or *Galegeae* or *Genisteeae* or *Hedysareae* or *Hypocalypteae* or *Indigoferaeae* or *Loteae* or *Millettieae* or *Mirbelieae* or *Phaseoleae* or
 10 *Podalyrieae* or *Psoraleeae* or *Robinieae* or *Sesbanieae* or *Sophoreae* or *Swartzieae* or *Thermopsidaeae* or *Trifolieae*.

In one embodiment, the plant based material from the sub-family *Papilionoideae* is from the tribe *Phaseoleae*, such as the genus *Adenodolichos* or *Alistilus* or *Amphicarpaea* or *Ancistrotropis* or *Apios* or *Atylosia* or *Bionia* or *Bolusafra* or *Butea* or *Cajanus* or *Calopogonium*
 15 or *Camptosema* or *Canavalia* or *Centrosema* or *Cleobulia* or *Clitoria* or *Cochlianthus* or *Cochlianthus* or *Collaea* or *Cologania* or *Condylostylis* or *Cratylia* or *Cymbosema* or *Decorsea* or *Dioclea* or *Dipogon* or *Dolichopsis* or *Dolichos* or *Dumasia* or *Dunbaria* or *Eriosema* or *Erythrina* or *Flemingia* or *Galactia* or *Glycine* or *Hardenbergia* or *Helicotropis* or *Kennedia* or *Lablab* or *Leptospron* or *Macroptilium* or *Macrotyloma* or *Mastersia* or *Mucuna* or *Mysanthus* or *Neonotonia*
 20 or *Neorautanenia* or *Nesphostylis* or *Nogra* or *Ophrestia* or *Otoptera* or *Oxyrhynchus* or *Pachyrhizus* or *Paracalyx* or *Phaseolus* or *Phylacium* or *Physostigma* or *Pseudeminia* or *Pseudovigna* or *Psophocarpus* or *Pueraria* or *Ramirezella* or *Rhodopis* or *Rhynchosia* or *Shuteria* or *Sigmoidotropis* or *Sinodolichos* or *Spathionema* or *Spatholobus* or *Sphenostylis* or *Strongylodon* or *Strophostyles* or *Teramnus* or *Teyleria* or *Vandasina* or *Vatovaea* or *Vigna* or
 25 *Wajira*.

In one embodiment, the plant based material from the sub-family *Papilionoideae* is from the genus *Glycine*, such as the species *Glycine aff. tabacina* or *Glycine albicans* or *Glycine aphyonota* or *Glycine arenaria* or *Glycine argyrea* or *Glycine canescens* or *Glycine clandestina* or *Glycine curvata* or *Glycine cyrtoloba* or *Glycine dolichocarpa* or *Glycine falcata* or *Glycine*
 30 *gracei* or *Glycine hirticaulis* or *Glycine lactovirens* or *Glycine latifolia* or *Glycine latrobeana* or *Glycine microphylla* or *Glycine peratosa* or *Glycine pindanica* or *Glycine pullenii* or *Glycine rubiginosa* or *Glycine stenophita* or *Glycine syndetika* or *Glycine tabacina* or *Glycine tomentella* or *Glycine sp. T1* or *Glycine sp. T5* or *Glycine gracilis* or *Glycine max* (soy bean) or *Glycine max* x *Glycine soja* or *Glycine soja* (wild soybean).

In one embodiment, the plant based material from the sub-family *Papilionoideae* is from the genus *Cajanus* such as the species *Cajanus cajan* (pigeon pea), *Cajanus cajanifolius* and *Cajanus scarabaeoide*.

In one embodiment, the plant based material from the sub-family *Papilionoideae* is from the genus *Phaseolus*, such as the species *Phaseolus acutifolius* (teparty bean) or *Phaseolus acutifolius* var. *latifolius* or *Phaseolus albescens* or *Phaseolus albiflorus* or *Phaseolus albinervus* or *Phaseolus altimontanus* or *Phaseolus amblyosepalus* or *Phaseolus angustissimus* or
5 *Phaseolus augusti* or *Phaseolus bolivianus* or *Phaseolus campanulatus* or *Phaseolus carteri* or *Phaseolus chiapasanus* or *Phaseolus coccineus* (scarlet runner bean) or *Phaseolus coccineus* subsp. *coccineus* or *Phaseolus coccineus* subsp. *polyanthus* or *Phaseolus costaricensis* or *Phaseolus dasycarpus* or *Phaseolus dumosus* or *Phaseolus esperanzae* or *Phaseolus esquincensis* or *Phaseolus filiformis* (slimjim bean) or *Phaseolus glabellus* or *Phaseolus*
10 *gladiolatus* or *Phaseolus grayanus* or *Phaseolus hintonii* or *Phaseolus jaliscanus* or *Phaseolus juquilensis* or *Phaseolus laxiflorus* or *Phaseolus leptostachyus* or *Phaseolus lignosus* or *Phaseolus lunatus* (lima bean) or *Phaseolus macrolepis* or *Phaseolus maculatifolius* or *Phaseolus maculatus* (cocolmecha bean) or *Phaseolus maculatus* subsp. *ritensis* or *Phaseolus macvaughii* or *Phaseolus magnilobatus* or *Phaseolus marechalii* or *Phaseolus micranthus* or *Phaseolus*
15 *microcarpus* or *Phaseolus mollis* or *Phaseolus neglectus* or *Phaseolus nelsonii* or *Phaseolus nodosus* or *Phaseolus novoleonensis* or *Phaseolus oaxacanus* or *Phaseolus oligospermus* or *Phaseolus pachyrrhizoides* or *Phaseolus parvifolius* or *Phaseolus parvulus* or *Phaseolus pauciflorus* or *Phaseolus pedicellatus* or *Phaseolus perplexus* or *Phaseolus persistentus* or *Phaseolus plagiocylix* or *Phaseolus pluriflorus* or *Phaseolus polymorphus* or *Phaseolus*
20 *polystachios* or *Phaseolus polystachios* subsp. *sinuatus* or *Phaseolus polystachios* subsp. *smilacifolius* or *Phaseolus reticulatus* or *Phaseolus rotundatus* or *Phaseolus salicifolius* or *Phaseolus sonorensis* or *Phaseolus talamancensis* or *Phaseolus tenellus* or *Phaseolus texensis* or *Phaseolus tuerckheimii* or *Phaseolus vulgaris* (French bean) or *Phaseolus vulgaris* var. *aborigineus* or *Phaseolus vulgaris* var. *nanus* or *Phaseolus xanthotrichus* or *Phaseolus xolocotzii*
25 or *Phaseolus zimapanensis*.

In one embodiment, the plant based material from the sub-family *Papilionoideae* is from the tribe *Cicereae*, such as the genus *Cicer*, such as the species *Cicer anatolicum* or *Cicer arietinum* (chickpea) or *Cicer bijugum* or *Cicer canariense* or *Cicer chorassanicum* or *Cicer cuneatum* or *Cicer echinospermum* or *Cicer flexuosum* or *Cicer floribundum* or *Cicer graecum* or
30 *Cicer incisum* or *Cicer isauricum* or *Cicer judaicum* or *Cicer kermanense* or *Cicer macracanthum* or *Cicer microphyllum* or *Cicer montbretii* or *Cicer multijugum* or *Cicer nuristanicum* or *Cicer oxyodon* or *Cicer pinnatifidum* or *Cicer pungens* or *Cicer rechingeri* or *Cicer reticulatum* or *Cicer songaricum* or *Cicer spiroceras* or *Cicer stapfianum* or *Cicer subaphyllum* or *Cicer tragacanthoides* or *Cicer yamashitae*

In one embodiment, the plant based material from the sub-family *Papilionoideae* is from the tribe *Genisteeae*, such as the genus *Adenocarpus* or *Anarthrophyllum* or *Argyrocytistus* or *Argyrolobium* or *Calicotome* or *Chamaecytistus* or *Cytisophyllum* or *Cytisus* or *Dichilus* or

Echinospartum or *Erinacea* or *Genista* or *Gonocytisus* or *Hesperolaburnum* or *Laburnum* or *Lembotropis* or *Lupinus* or *Melolobium* or *Petteria* or *Podocytisus* or *Polhillia* or *Retama* or *Sellocharis* or *Spartium* or *Stauracanthus* or *Teline* or *Ulex*

5 In one embodiment, the plant based material from the sub-family *Papilionoideae* is from the tribe *Fabeae*, such as the genus *Lathyrus* or *Lens* or *Pisum* or *Vavilovia* or *Vicia*. In one embodiment, the plant based material from the sub-family *Papilionoideae* is from the genus *Lens*, such as the species *Lens culinaris* (lentil) or *Lens culinaris subsp. culinaris* or *Lens culinaris subsp. odemensis* or *Lens culinaris subsp. tomentosus* or *Lens cyanea* or *Lens ervoides* or *Lens lamottei* or *Lens nigricans* or *Lens orientalis* (ye bing dou).

10 In one embodiment, the plant based material from the sub-family *Papilionoideae* is from the genus *Vicia*, such as the species *Vicia garinensis* or *Vicia sojakii* or *Vicia rechingeri* or *Vicia kurdica* or *Vicia multijuga* or *Vicia akhmaganica* or *Vicia variabilis* or *Vicia variegata* or *Vicia persica* or *Vicia kotschyana* or *Vicia hirta* or *Vicia gregaria* or *Vicia ciceroidea* or *Vicia cappadocica* or *Vicia balansae* or *Vicia aucheri* or *Vicia sp. 'telaponensis'* or *Vicia venulosa* or *Vicia subvillosa*
 15 or *Vicia stenophylla* or *Vicia sicula* or *Vicia sibthorpii* or *Vicia semiglabra* or *Vicia scandens* or *Vicia pinetorum* or *Vicia picta* or *Vicia pectinata* or *Vicia paucifolia* or *Vicia palaestina* or *Vicia onobrychioides* or *Vicia ochroleuca* or *Vicia nataliae* or *Vicia montevidensis* or *Vicia monardii* or *Vicia minutiflora* or *Vicia menziesii* or *Vicia megalotropis* or *Vicia malosana* or *Vicia lunata* or *Vicia leucantha* or *Vicia leavenworthii* or *Vicia larissae* or *Vicia iranica* or *Vicia incana* or *Vicia hololasia*
 20 or *Vicia glauca* or *Vicia freyniana* or *Vicia floridana* or *Vicia filicaulis* or *Vicia ferreirensis* or *Vicia exigua* or *Vicia dennesiana* or *Vicia cypria* or *Vicia cretica* or *Vicia costata* or *Vicia claessensii* or *Vicia chaetocalyx* or *Vicia cassia* or *Vicia capreolata* or *Vicia caesarea* or *Vicia biennis* or *Vicia baicalensis* or *Vicia altissima* or *Vicia alpestris* or *Vicia acutifolia* or *Vicia pubescens* or *Vicia cirrhosa* or *Vicia koeieana* or *Vicia ramuliflora* or *Vicia multicaulis* or *Vicia parviflora* or *Vicia*
 25 *vicioides* or *Vicia tenuifolia* or *Vicia orobus* or *Vicia nigra* or *Vicia incisa* or *Vicia epetiolaris* or *Vicia crocea* or *Vicia sparsiflora* or *Vicia nummularia* or *Vicia dichroantha* or *Vicia cassubica* or *Vicia monantha* (bard vetch) or *Vicia cinerea* or *Vicia oroboides* or *Vicia tibetica* or *Vicia caroliniana* (Carolina or wood vetch) or *Vicia disperma* or *Vicia esdraelonensis* or *Vicia pulchella* or *Vicia mexicana* or *Vicia leucophaea* or *Vicia humilis* or *Vicia barbazitae* or *Vicia pyrenaica* or *Vicia*
 30 *qatmensis* or *Vicia lathyroides* or *Vicia cuspidata* or *Vicia dionysiensis* or *Vicia abbreviata* or *Vicia sepium* or *Vicia sericocarpa* or *Vicia noeana* or *Vicia hyrcanica* or *Vicia hybrida* or *Vicia galeata* or *Vicia ciliatula* or *Vicia assyriaca* or *Vicia tigridis* or *Vicia anatolica* or *Vicia sylvatica* or *Vicia dumetorum* or *Vicia mollis* or *Vicia aintabensis* or *Vicia peregrina* or *Vicia lutea* (yellow vetch) or *Vicia grandiflora* or *Vicia articulata* or *Vicia americana* or *Vicia michauxii* or *Vicia vicina* or *Vicia*
 35 *venosa* or *Vicia tetrasperma* or *Vicia ervilia* or *Vicia benghalensis* (purple or winter vetch) or *Vicia angustipinnata* or *Vicia amurensis* or *Vicia unijuga* or *Vicia pseudo-orobus* or *Vicia pisiformis* or *Vicia nipponica* or *Vicia nigricans* or *Vicia linearifolia* or *Vicia japonica* or *Vicia hirticalycina* or *Vicia*

fauriae or *Vicia chosenensis* or *Vicia bungei* or *Vicia bifolia* or *Vicia amoena* or *Vicia montbretii* or *Vicia serratifolia* or *Vicia paucijuga* or *Vicia kalakhensis* or *Vicia johannis* or *Vicia hyaeniscyamus* or *Vicia galilaea* or *Vicia eristalioides* or *Vicia bithynica* or *Vicia melanops* or *Vicia ludoviciana* or *Vicia pannonica* or *Vicia narbonensis* or *Vicia villosa* or *Vicia hirsuta* or *Vicia sativa* (spring vetch)
 5 or *Vicia faba* (broad bean or fava bean) or *Vicia cracca* (bird vetch).

In one embodiment, the plant based material from the sub-family *Papilionoideae* is from the genus *Pisum*, such as the species *Pisum abyssinicum* (Abyssinian pea) or *Pisum fulvum* or *Pisum sativum* (pea) or *Pisum sativum* subsp. *asiaticum* or *Pisum sativum* subsp. *elatius* (wild pea) or *Pisum sativum* var. *pumilio* (Syrian fodder pea) or *Pisum sativum* subsp. *jomardii* or *Pisum*
 10 *sativum* subsp. *Sativum* or *Pisum sativum* var. *arvense* or *Pisum sativum* var. *choresmicum* or *Pisum sativum* var. *macrocarpon* (snow pea) or *Pisum sativum* var. *ponderosum* or *Pisum sativum* var. *tibeticum* or *Pisum sativum* subsp. *transcausicum*

In one embodiment, the plant based material from the sub-family *Papilionoideae* is from the tribe *Dalbergieae*, such as the genus *Adesmia* or *Aeschynomene* or *Amicia* or *Andira* or
 15 *Arachis* or *Brya* or *Bryaspis* or *Cascanonia* or *Centrolobium* or *Chaetocalyx* or *Chapmannia* or *Cranocarpus* or *Cyclocarpa* or *Dalbergia* or *Diphysa* or *Discolobium* or *Etaballia* or *Fiebrigiella* or *Fissicalyx* or *Geissaspis* or *Geoffroea* or *Grazilodendron* or *Humularia* or *Hymenolobium* or *Inocarpus* or *Kotschya* or *Machaerium* or *Maraniona* or *Nissolia* or *Ormocarpopsis* or *Ormocarpum* or *Paramachaerium* or *Peltiera* or *Pictetia* or *Platymiscium* or *Platypodium* or
 20 *Poiretia* or *Pterocarpus* or *Ramorinoa* or *Riedeliella* or *Smithia* or *Soemmeringia* or *Steinbachiella* or *Stylosanthes* or *Tipuana* or *Weberbauerella* or *Zornia*.

In one embodiment, the plant based material from the sub-family *Papilionoideae* is from the genus *Arachis*, such as the species *Appressipila* (amendoim bravo) or *Arachis batizocoi* or *Arachis brevipetiolata* or *Arachis burchellii* or *Arachis burkartii* or *Arachis cardenasii* or *Arachis*
 25 *chiquitana* or *Arachis correntina* or *Arachis cruziana* or *Arachis decora* or *Arachis diogoi* or *Arachis duranensis* or *Arachis duranensis* x *Arachis stenosperma* or *Arachis glabrata* (amendoim-bravo) or *Arachis glabrata* var. *glabrata* or *Arachis glabrata* var. *hagenbeckii* or *Arachis glabrata* x *Arachis hypogaea* or *Arachis glandulifera* or *Arachis guaranítica* or *Arachis helodes* or *Arachis hermannii* or *Arachis hoehnei* or *Arachis hypogaea* (peanut) or *Arachis hypogaea* subsp.
 30 *Fastigiata* or *Arachis hypogaea* var. *vulgaris* (Spanish peanut) or *Arachis hypogaea* subsp. *Hypogaea* or *Arachis hypogaea* var. *hirsuta* or *Arachis ipaensis* or *Arachis ipaensis* x *Arachis magna* or *Arachis kempff-mercadoi* or *Arachis kretschmeri* or *Arachis kuhlmannii* or *Arachis linearifolia* or *Arachis lutescens* or *Arachis magna* or *Arachis major* or *Arachis matiensis* or *Arachis microsperma* or *Arachis monticola* or *Arachis palustris* or *Arachis paraguariensis* or
 35 *Arachis paraguariensis* subsp. *capibarensis* or *Arachis paraguariensis* subsp. *paraguariensis* or *Arachis pflugeae* or *Arachis pintoi* or *Arachis praecox* or *Arachis pusilla* (amendoim de caracar) or *Arachis repens* or *Arachis rigonii* or *Arachis schinini* or *Arachis simpsonii* or *Arachis stenophylla*

or *Arachis stenosperma* or *Arachis stenosperma* x *Arachis cardenasii* or *Arachis sylvestris* (amendoim do porco) or *Arachis trinitensis* or *Arachis triseminata* or *Arachis tuberosa* or *Arachis valida* or *Arachis villosa* or *Arachis villosulicarpa* or *Arachis williamsii*.

In one aspect, the plant based material is from the taxonomic order *Brassicales*, such as
5 the family *Brassicaceae*, preferably the tribe *Brassiceae*, more preferably the family *Brassica*.

In one embodiment, the plant based material from the tribe *Brassiceae* is from the family *Brassica*, such as *Brassica aucheri*, *Brassica balearica*, *Brassica barrelieri*, *Brassica carinata* (Abyssinian mustard), *Brassica carinata* x *Brassica napus*, *Brassica carinata* x *Brassica rapa*,
10 *Brassica cretica*, *Brassica deflexa*, *Brassica desnottesii*, *Brassica drepanensis*, *Brassica elongata*, *Brassica fruticulosa*, *Brassica fruticulosa* subsp. *cossoniana*, *Brassica fruticulosa* subsp. *mauritanica*, *Brassica fruticulosa* subsp. *rifana*, *Brassica gravinae*, *Brassica hilarionis*, *Brassica hybrid cultivar*, *Brassica incana*, *Brassica insularis*, *Brassica insularis* subsp. *insularis*, *Brassica juncea* (Indian mustard), *Brassica juncea* var. *crassicaulis*, *Brassica juncea* var. *gemmaifera*, *Brassica juncea* var. *gracilis*, *Brassica juncea* var. *juncea*, *Brassica juncea* var. *multiceps*,
15 *Brassica juncea* var. *multisecta*, *Brassica juncea* var. *napiformis* (jie cai ge da), *Brassica juncea* var. *rugosa*, *Brassica juncea* var. *strumata*, *Brassica juncea* var. *subintegrifolia*, *Brassica juncea* var. *tumida* (zha cai), *Brassica juncea* var. *utilis*, *Brassica macrocarpa*, *Brassica maurorum*, *Brassica montana*, *Brassica napus* (rape), *Brassica napus* subsp. *rapifera* (Swedish turnip), *Brassica napus* var. *napus* (annual rape), *Brassica napus* x *Brassica rapa*, *Brassica nigra* (black mustard), *Brassica nigra* var. *abyssinica*, *Brassica oleracea*, *Brassica oleracea* var. *albiflora*,
20 *Brassica oleracea* var. *alboglabra* (Chinese kale), *Brassica oleracea* var. *botrytis* (cauliflower), *Brassica oleracea* var. *capitata* (cabbage), *Brassica oleracea* var. *costata* (Bedford cabbage), *Brassica oleracea* var. *gemmaifera* (Brussels sprouts), *Brassica oleracea* var. *gongylodes* (kohlrabi), *Brassica oleracea* var. *italica* (asparagus broccoli), *Brassica oleracea* var. *medullosa* (marrow-stem kale), *Brassica oleracea* var. *oleracea*, *Brassica oleracea* var. *ramosa* (branching bush kale), *Brassica oleracea* var. *sabauda*, *Brassica oleracea* var. *viridis* (kale), *Brassica oleracea* x *Brassica rapa* subsp. *pekinensis*, *Brassica oxyrrhina*, *Brassica procumbens*, *Brassica rapa* (field mustard), *Brassica rapa* subsp. *chinensis* (bok-choy), *Brassica rapa* var. *parachinensis* (cai xin), *Brassica rapa* var. *purpuraria* (purple stem mustard), *Brassica rapa* subsp. *narinosa*,
30 *Brassica rapa* subsp. *nipposinica* (mizuna), *Brassica rapa* var. *perviridis* (kabuna), *Brassica rapa* subsp. *oleifera* (biennial turnip rape), *Brassica rapa* (Nippo-oleifera Group), *Brassica rapa* subsp. *pekinensis* (Chinese cabbage), *Brassica rapa* subsp. *rapa* (turnip), *Brassica rapa* var. *oleifera*, *Brassica rapa* x *Brassica nigra*, *Brassica repanda*, *Brassica repanda* subsp. *baldensis*, *Brassica repanda* subsp. *blancoana*, *Brassica repanda* subsp. *cadevallii*, *Brassica repanda* subsp. *confusa*, *Brassica repanda* subsp. *glabrescens*, *Brassica repanda* subsp. *gypsicola*, *Brassica repanda* subsp. *latisiliqua*, *Brassica repanda* subsp. *maritima*, *Brassica repanda* subsp. *repanda*, *Brassica repanda* subsp. *saxatilis*, *Brassica rupestris*, *Brassica ruvo*

(broccoletto), *Brassica souliei*, *Brassica souliei* subsp. *amplexicaulis*, *Brassica spinescens*, *Brassica tournefortii*, *Brassica villosa* or *Brassica villosa* subsp. *Bivoniana*.

In particular embodiments, the plant based material is soybean, wild soybean, beans, lupin, tepary bean, scarlet runner bean, slimjim bean, lima bean, French bean, Broad bean (fava bean), chickpea, lentil, peanut, Spanish peanut, canola, rapeseed (oilseed rape) or pea or in a processed form such as soybean meal, full fat soy bean meal, soy protein concentrate (SPC), fermented soybean meal (FSBM) or any combination thereof. In a preferred embodiment, the plant based material is soybean or soybean meal.

Animal Feed and Animal Feed Additives

The present invention also relates to animal feed compositions and animal feed additives comprising one or more alpha-galactosidases of the invention. In an embodiment, the animal feed or animal feed additive comprises a formulating agent and one or more alpha-galactosidases of the invention. In a further embodiment, the formulating agent comprises one or more of the following compounds: glycerol, ethylene glycol, 1, 2-propylene glycol or 1, 3-propylene glycol, sodium chloride, sodium benzoate, potassium sorbate, sodium sulfate, potassium sulfate, magnesium sulfate, sodium thiosulfate, calcium carbonate, sodium citrate, dextrin, glucose, sucrose, sorbitol, lactose, starch, kaolin and cellulose.

Animal feed compositions or diets have a relatively high content of protein. Poultry and pig diets can be characterised as indicated in Table B of WO 01/58275, columns 2-3. Fish diets can be characterised as indicated in column 4 of this Table B. Furthermore such fish diets usually have a crude fat content of 200-310 g/kg.

An animal feed composition according to the invention has a crude protein content of 50-800 g/kg, and furthermore comprises at least one alpha-galactosidase as claimed herein.

Furthermore, or in the alternative (to the crude protein content indicated above), the animal feed composition of the invention has a content of metabolisable energy of 10-30 MJ/kg; and/or a content of calcium of 0.1-200 g/kg; and/or a content of available phosphorus of 0.1-200 g/kg; and/or a content of methionine of 0.1-100 g/kg; and/or a content of methionine plus cysteine of 0.1-150 g/kg; and/or a content of lysine of 0.5-50 g/kg.

In particular embodiments, the content of metabolisable energy, crude protein, calcium, phosphorus, methionine, methionine plus cysteine, and/or lysine is within any one of ranges 2, 3, 4 or 5 in Table B of WO 01/58275 (R. 2-5).

Crude protein is calculated as nitrogen (N) multiplied by a factor 6.25, i.e. Crude protein (g/kg)= N (g/kg) x 6.25. The nitrogen content is determined by the Kjeldahl method (A.O.A.C., 1984, Official Methods of Analysis 14th ed., Association of Official Analytical Chemists, Washington DC).

Metabolisable energy can be calculated on the basis of the NRC publication Nutrient requirements in swine, ninth revised edition 1988, subcommittee on swine nutrition, committee

on animal nutrition, board of agriculture, national research council. National Academy Press, Washington, D.C., pp. 2-6, and the European Table of Energy Values for Poultry Feed-stuffs, Spelderholt centre for poultry research and extension, 7361 DA Beekbergen, The Netherlands. Grafisch bedrijf Ponsen & looijen bv, Wageningen. ISBN 90-71463-12-5.

5 The dietary content of calcium, available phosphorus and amino acids in complete animal diets is calculated on the basis of feed tables such as Veevoedertabel 1997, gegevens over chemische samenstelling, verteerbaarheid en voederwaarde van voedermiddelen, Central Veevoederbureau, Runderweg 6, 8219 pk Lelystad. ISBN 90-72839-13-7.

10 In a particular embodiment, the animal feed composition of the invention contains at least one vegetable protein as defined above.

The animal feed composition of the invention may also contain animal protein, such as Meat and Bone Meal, Feather meal, and/or Fish Meal, typically in an amount of 0-25%. The animal feed composition of the invention may also comprise Dried Distillers Grains with Solubles (DDGS), typically in amounts of 0-30%.

15 The animal feed composition of the invention may also contain insect protein, such as protein from mealworm, housefly or black soldier fly larvae, typically in meal form. Insect meal may replace fishmeal entirely or in part, and thus may constitute 0-10% of the total feed.

20 In still further particular embodiments, the animal feed composition of the invention contains 0-80% maize; and/or 0-80% sorghum; and/or 0-70% wheat; and/or 0-70% barley; and/or 0-30% oats; and/or 0-40% soybean meal; and/or 0-25% fish meal; and/or 0-25% meat and bone meal; and/or 0-20% whey.

25 The animal feed may comprise vegetable proteins. In particular embodiments, the protein content of the vegetable proteins is at least 10, 20, 30, 40, 50, 60, 70, 80, or 90% (w/w). Vegetable proteins may be derived from vegetable protein sources, such as legumes and cereals, for example, materials from plants of the families *Fabaceae* (*Leguminosae*), *Brassicaceae*, *Amaranthaceae*, and *Poaceae*, such as soybean meal, lupin meal, rapeseed meal, and combinations thereof.

30 In a particular embodiment, the vegetable protein source is material from one or more plants of the family *Fabaceae*, e.g., soybean, lupine, pea, or bean. In another particular embodiment, the vegetable protein source is material from one or more plants of the family *Amaranthaceae*, e.g. beet, sugar beet, spinach or quinoa. Other examples of vegetable protein sources are rapeseed, crambe and cabbage. In another particular embodiment, soybean is a preferred vegetable protein source. Other examples of vegetable protein sources are cereals such as barley, wheat, rye, oat, maize (corn), rice, and sorghum.

35 Animal diets can e.g. be manufactured as mash feed (non-pelleted) or pelleted feed. Typically, the milled feed-stuffs are mixed and sufficient amounts of essential vitamins and minerals are added according to the specifications for the species in question. Enzymes can be

added as solid or liquid enzyme formulations. For example, for mash feed a solid or liquid enzyme formulation may be added before or during the ingredient mixing step. For pelleted feed the (liquid or solid) alpha-galactosidase/enzyme preparation may also be added before or during the feed ingredient step. Typically a liquid alpha-galactosidase/enzyme preparation comprises the alpha-galactosidase of the invention optionally with a polyol, such as glycerol, ethylene glycol or propylene glycol, and is added after the pelleting step, such as by spraying the liquid formulation onto the pellets. The enzyme may also be incorporated in a feed additive or premix.

Alternatively, the alpha-galactosidase can be prepared by freezing a mixture of liquid enzyme solution with a bulking agent such as ground soybean meal, and then lyophilizing the mixture.

In an embodiment, the animal feed or animal feed additive comprises one or more additional enzymes. In an embodiment, the animal feed comprises one or more microbes. In an embodiment, the animal feed comprises one or more vitamins. In an embodiment, the animal feed comprises one or more minerals. In an embodiment, the animal feed comprises one or more amino acids. In an embodiment, the animal feed comprises one or more other feed ingredients.

In another embodiment, the animal feed or animal feed additive comprises the polypeptide of the invention, one or more formulating agents and one or more additional enzymes. In an embodiment, the animal feed or animal feed additive comprises the polypeptide of the invention, one or more formulating agents and one or more microbes. In an embodiment, the animal feed comprises the polypeptide of the invention, one or more formulating agents and one or more vitamins. In an embodiment, the animal feed or animal feed additive comprises one or more minerals. In an embodiment, the animal feed or animal feed additive comprises the polypeptide of the invention, one or more formulating agents and one or more amino acids. In an embodiment, the animal feed or animal feed additive comprises the polypeptide of the invention, one or more formulating agents and one or more other feed ingredients.

In a further embodiment, the animal feed or animal feed additive comprises the polypeptide of the invention, one or more formulating agents and one or more components selected from the list consisting of: one or more additional enzymes; one or more microbes; one or more vitamins; one or more minerals; one or more amino acids; and one or more other feed ingredients.

In an embodiment, the animal feed additive comprises one or more formulating agents, preferably as described herein below.

In an embodiment, the animal feed additive comprises one or more additional enzymes, preferably as described herein below.

In an embodiment, the animal feed additive comprises one or more probiotics, preferably as described herein below.

In an embodiment, the animal feed additive comprises one or more vitamins, preferably as described herein below.

In an embodiment, the animal feed additive comprises one or more minerals, preferably as described herein below.

5 In an embodiment, the animal feed additive comprises one or more amino acids, preferably as described herein below.

In an embodiment, the animal feed additive comprises one or more prebiotics, preferably as described herein below.

10 In an embodiment, the animal feed additive comprises one or more organic acids, preferably as described herein below.

In an embodiment, the animal feed additive comprises one or more phytochemicals, preferably as described herein below.

The final enzyme concentration in the diet is within the range of 0.01-200 mg enzyme protein per kg diet, preferably between 0.1-100 mg/kg diet, more preferably 0.5-50 mg, even more
15 preferably 1-25 mg enzyme protein per kg animal diet.

It is at present contemplated that the enzyme is administered in one or more of the following amounts (dosage ranges): 0.01-200; 0.05-100; 0.1-50; 0.2-20; 0.1-1; 0.2-2; 0.5-5; 5-50; 10-30; 20-30; 10-20; 15-25; or 1-10; – all these ranges being in mg alpha-galactosidase protein per kg feed (ppm).

20 For determining mg alpha-galactosidase protein per kg feed, the alpha-galactosidase is purified from the feed composition, and the specific activity of the purified alpha-galactosidase is determined using a relevant assay (see under alpha-galactosidase activity). The alpha-galactosidase activity of the feed composition as such is also determined using the same assay, and on the basis of these two determinations, the dosage in mg alpha-galactosidase protein per
25 kg feed is calculated.

In a particular embodiment, the animal feed additive of the invention is intended for being included (or prescribed as having to be included) in animal diets or feed at levels of 0.01 to 10.0%; more particularly 0.05 to 5.0%; or 0.2 to 1.0% (% meaning g additive per 100 g feed). This is so in particular for premixes.

30 The same principles apply for determining mg alpha-galactosidase protein in feed additives. Of course, if a sample is available of the alpha-galactosidase used for preparing the feed additive or the feed, the specific activity is determined from this sample (no need to purify the alpha-galactosidase from the feed composition or the additive).

Additional Enzymes

35 In another embodiment, the compositions described herein optionally include one or more enzymes. Enzymes can be classified on the basis of the handbook Enzyme Nomenclature from NC-IUBMB, 1992), see also the ENZYME site at the internet: <http://www.expasy.ch/enzyme/>.

ENZYME is a repository of information relative to the nomenclature of enzymes. It is primarily based on the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUB-MB), Academic Press, Inc., 1992, and it describes each type of characterized enzyme for which an EC (Enzyme Commission) number has been provided
5 (Bairoch A. The ENZYME database, 2000, *Nucleic Acids Res* 28:304-305). This IUB-MB Enzyme nomenclature is based on their substrate specificity and occasionally on their molecular mechanism; such a classification does not reflect the structural features of these enzymes.

Another classification of certain glycoside hydrolase enzymes, such as endoglucanase, galactanase, mannanase, dextranase, lysozyme and galactosidase is described in Henrissat *et al*, "The carbohydrate-active enzymes database (CAZy) in 2013", *Nucl. Acids Res.* (1 January
10 2014) 42 (D1): D490-D495; see also www.cazy.org.

Thus the composition of the invention may also comprise at least one other enzyme selected from the group comprising of acetylxyloxyesterase (EC 3.1.1.23), acylglycerol lipase (EC 3.1.1.72), alpha-amylase (EC 3.2.1.1), beta-amylase (EC 3.2.1.2), arabinofuranosidase (EC
15 3.2.1.55), cellobiohydrolases (EC 3.2.1.91), cellulase (EC 3.2.1.4), feruloyl esterase (EC 3.1.1.73), galactanase (EC 3.2.1.89), alpha-galactosidase (EC 3.2.1.22), beta-galactosidase (EC 3.2.1.23), beta-glucanase (EC 3.2.1.6), beta-glucosidase (EC 3.2.1.21), triacylglycerol lipase (EC 3.1.1.3), lysophospholipase (EC 3.1.1.5), lysozyme (EC 3.2.1.17), alpha-mannosidase (EC 3.2.1.24), beta-mannosidase (mannanase) (EC 3.2.1.25), phytase (EC 3.1.3.8, EC 3.1.3.26, EC
20 3.1.3.72), phospholipase A1 (EC 3.1.1.32), phospholipase A2 (EC 3.1.1.4), phospholipase D (EC 3.1.4.4), protease (EC 3.4), pullulanase (EC 3.2.1.41), pectinesterase (EC 3.1.1.11), xylanase (EC 3.2.1.8, EC 3.2.1.136), beta-xylosidase (EC 3.2.1.37), or any combination thereof.

In a particular embodiment, the composition of the invention comprises a galactanase (EC 3.2.1.89) and a beta-galactosidase (EC 3.2.1.23).

In a particular embodiment, the composition of the invention comprises a phytase (EC 3.1.3.8 or 3.1.3.26). Examples of commercially available phytases include Bio-Feed™ Phytase (Novozymes), Ronozyme® P, Ronozyme® NP and Ronozyme® HiPhos (DSM Nutritional Products), Natuphos™ (BASF), Natuphos™ E (BASF), Finase® and Quantum® Blue (AB Enzymes), OptiPhos® (Huvepharma), AveMix® Phytase (Aveve Biochem), Phyzyme® XP
30 (Verenium/DuPont) and Aextra® PHY (DuPont). Other preferred phytases include those described in e.g. WO 98/28408, WO 00/43503, and WO 03/066847.

In a particular embodiment, the composition of the invention comprises a xylanase (EC 3.2.1.8 or EC 3.2.1.136). Examples of commercially available xylanases include Ronozyme® WX (DSM Nutritional Products), Econase® XT and Barley (AB Vista), Xylathin® (Verenium),
35 Hostazym® X (Huvepharma), Aextra® XB (Xylanase/beta-glucanase, DuPont) and Aextra® XAP (Xylanase/amylase/protease, DuPont), AveMix® XG 10 (xylanase/glucanase) and AveMix® 02 CS (xylanase/glucanase/pectinase, Aveve Biochem), and Naturgrain (BASF).

In a particular embodiment, the composition of the invention comprises a protease (EC 3.4). Examples of commercially available proteases include Ronozyme® ProAct (DSM Nutritional Products).

5 In a particular embodiment, the composition of the invention comprises an alpha-amylase (EC 3.2.1.1). Examples of commercially available alpha-amylases include Ronozyme® A and RONOZYME® RumiStar™ (DSM Nutritional Products).

10 In one embodiment, the composition of the invention comprises a multicomponent enzyme product, such as FRA® Octazyme (Framelco), Ronozyme® G2, Ronozyme® VP and Ronozyme® MultiGrain (DSM Nutritional Products), Rovabio® Excel or Rovabio® Advance (Adisseo).

Eubiotics

15 Eubiotics are compounds which are designed to give a healthy balance of the micro-flora in the gastrointestinal tract. Eubiotics cover a number of different feed additives, such as probiotics, prebiotics, phytochemicals (essential oils) and organic acids which are described in more detail below.

Probiotics

20 In an embodiment, the animal feed composition further comprises one or more additional probiotic. In a particular embodiment, the animal feed composition further comprises a bacterium from one or more of the following genera: *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Bacillus*, *Pediococcus*, *Enterococcus*, *Leuconostoc*, *Carnobacterium*, *Propionibacterium*, *Bifidobacterium*, *Clostridium* and *Megasphaera* or any combination thereof.

25 In a preferred embodiment, animal feed composition further comprises a bacterium from one or more of the following strains: *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus pumilus*, *Bacillus polymyxa*, *Bacillus megaterium*, *Bacillus coagulans*, *Bacillus circulans*, *Enterococcus faecium*, *Enterococcus spp*, and *Pediococcus spp*, *Lactobacillus spp*, *Bifidobacterium spp*, *Lactobacillus acidophilus*, *Pediococcus acidilactici*, *Lactococcus lactis*, *Bifidobacterium bifidum*, *Propionibacterium thoenii*, *Lactobacillus farciminus*, *lactobacillus rhamnosus*, *Clostridium butyricum*, *Bifidobacterium animalis ssp. animalis*, *Lactobacillus reuteri*, *Lactobacillus salivarius ssp. salivarius*, *Megasphaera elsdenii*,
30 *Propionibacteria sp.*

35 In a more preferred embodiment, composition, animal feed additive or animal feed further comprises a bacterium from one or more of the following strains of *Bacillus subtilis*: 3A-P4 (PTA-6506), 15A-P4 (PTA-6507), 22C-P1 (PTA-6508), 2084 (NRRL B-500130), LSSA01 (NRRL-B-50104), BS27 (NRRL B-501 05), BS 18 (NRRL B-50633), BS 278 (NRRL B-50634), DSM 29870, DSM 29871, DSM 32315, NRRL B-50136, NRRL B-50605, NRRL B-50606, NRRL B-50622 and PTA-7547.

In a more preferred embodiment, composition, animal feed additive or animal feed further comprises a bacterium from one or more of the following strains of *Bacillus pumilus*: NRRL B-50016, ATCC 700385, NRRL B-50885 or NRRL B-50886.

5 In a more preferred embodiment, composition, animal feed additive or animal feed further comprises a bacterium from one or more of the following strains of *Bacillus licheniformis*: NRRL B-50015, NRRL B-50621 or NRRL B-50623.

In a more preferred embodiment, composition, animal feed additive or animal feed further comprises a bacterium from one or more of the following strains of *Bacillus amyloliquefaciens*: DSM 29869, DSM 29869, NRRL B-50607, PTA-7543, PTA-7549, NRRL B-50349, NRRL B-50606, NRRL B-50013, NRRL B-50151, NRRL B-50141, NRRL B-50147 or NRRL B-50888.

The bacterial count of each of the bacterial strains in the animal feed composition is between 1×10^4 and 1×10^{14} CFU/kg of dry matter, preferably between 1×10^6 and 1×10^{12} CFU/kg of dry matter, and more preferably between 1×10^7 and 1×10^{11} CFU/kg of dry matter. In a more preferred embodiment the bacterial count of each of the bacterial strains in the animal feed composition is between 1×10^8 and 1×10^{10} CFU/kg of dry matter.

The bacterial count of each of the bacterial strains in the animal feed composition is between 1×10^5 and 1×10^{15} CFU/animal/day, preferably between 1×10^7 and 1×10^{13} CFU/animal/day, and more preferably between 1×10^8 and 1×10^{12} CFU/animal/day. In a more preferred embodiment the bacterial count of each of the bacterial strains in the animal feed composition is between 1×10^9 and 1×10^{11} CFU/animal/day.

In another embodiment, the one or more bacterial strains are present in the form of a stable spore.

Examples of commercial products are Cylactin® (DSM Nutritional Products), Alterion (Adisseo), Enviva PRO (DuPont Animal Nutrition), Gallipro®, Gallipro® Max, Probios® Guard, Lactiferm® and Bioplus® (Chr Hansen), PoultryStar®, PoultryStar® sol, PoultryStar® me, AquaStar® (Biomim), Syncra® (mix enzyme + probiotic, DuPont Animal Nutrition), Ecobiol® and Fecinor® (Norel/Evonik) and GutCare® PY1 (Evonik).

Prebiotics

Prebiotics are substances that induce the growth or activity of microorganisms (e.g., bacteria and fungi) that contribute to the well-being of their host. Prebiotics are typically non-digestible fiber compounds that pass undigested through the upper part of the gastrointestinal tract and stimulate the growth or activity of advantageous bacteria that colonize the large bowel by acting as substrate for them. Normally, prebiotics increase the number or activity of bifidobacteria and lactic acid bacteria in the GI tract.

35 Yeast derivatives (inactivated whole yeasts or yeast cell walls) can also be considered as prebiotics. They often comprise mannan-oligosaccharids, yeast beta-glucans or protein contents and are normally derived from the cell wall of the yeast, *Saccharomyces cerevisiae*.

Examples of yeast products are Yang® and Agrimos (Lallemand Animal Nutrition).

Phytogenics

Phytogenics are a group of natural growth promoters or non-antibiotic growth promoters used as feed additives, derived from herbs, spices or other plants. Phytogenics can be single substances prepared from essential oils/extracts, essential oils/extracts, single plants and mixture of plants (herbal products) or mixture of essential oils/extracts/plants (specialized products).

Examples of phytogenics are rosemary, sage, oregano, thyme, clove, and lemongrass. Examples of essential oils are thymol, eugenol, meta-cresol, vaniline, salicylate, resorcinol, guajacol, gingerol, lavender oil, ionones, irone, eucalyptol, menthol, peppermint oil, alpha-pinene; limonene, anethol, linalool, methyl dihydrojasmonate, carvacrol, propionic acid/propionate, acetic acid/acetate, butyric acid/butyrate, rosemary oil, clove oil, geraniol, terpineol, citronellol, amyl and/or benzyl salicylate, cinnamaldehyde, plant polyphenol (tannin), turmeric and curcuma extract.

Examples of commercial products are Crina® (DSM Nutritional Products); Cinergy™, Cinergy™ FIT, Biacid™, (Cargill), Digestarom® and Digestarom® DC (Biomim) and Envivo EO (DuPont Animal Nutrition).

Organic Acids

Organic acids (C1–C7) are widely distributed in nature as normal constituents of plants or animal tissues. They are also formed through microbial fermentation of carbohydrates mainly in the large intestine. They are often used in swine and poultry production as a replacement of antibiotic growth promoters since they have a preventive effect on the intestinal problems like necrotic enteritis in chickens and *Escherichia coli* infection in young pigs. Organic acids can be sold as mono component or mixtures of typically 2 or 3 different organic acids. Examples of organic acids are propionic acid, formic acid, citric acid, lactic acid, sorbic acid, malic acid, acetic acid, fumaric acid, benzoic acid, butyric acid and tartaric acid or their salt (typically sodium or potassium salt such as potassium diformate or sodium butyrate).

Examples of commercial products are VevoVital® (DSM Nutritional Products), Amasil®, Luprisil®, Lupro-Grain®, Lupro-Cid®, Lupro-Mix®, Lupro-Mix® NA (BASF), n-Butyric Acid AF (OXEA), Biacid™, Prohacid™ Classic and Prohacid™ Advance™ (Cargill), Biotronic® (Biomim) and Adimix Precision (Nutriad).

Premix

The incorporation of the composition of feed additives as exemplified herein above to animal feeds, for example poultry feeds, is in practice carried out using a concentrate or a premix. A premix designates a preferably uniform mixture of one or more microingredients with diluent and/or carrier. Premixes are used to facilitate uniform dispersion of micro-ingredients in a larger

mix. A premix according to the invention can be added to feed ingredients or to the drinking water as solids (for example as water soluble powder) or liquids.

Amino Acids

The composition of the invention may further comprise one or more amino acids.
5 Examples of amino acids which are used in animal feed are lysine, alanine, beta-alanine, threonine, methionine and tryptophan.

Vitamins and Minerals

In another embodiment, the animal feed may include one or more vitamins, such as one or more fat-soluble vitamins and/or one or more water-soluble vitamins. In another embodiment,
10 the animal feed may optionally include one or more minerals, such as one or more trace minerals and/or one or more macro minerals.

Usually fat- and water-soluble vitamins, as well as trace minerals form part of a so-called premix intended for addition to the feed, whereas macro minerals are usually separately added to the feed.

15 Non-limiting examples of fat-soluble vitamins include vitamin A, vitamin D3, vitamin E, and vitamin K, *e.g.*, vitamin K3.

Non-limiting examples of water-soluble vitamins include vitamin B12, biotin and choline, vitamin B1, vitamin B2, vitamin B6, niacin, folic acid and panthothenate, *e.g.*, Ca-D-panthothenate.

20 Non-limiting examples of trace minerals include boron, cobalt, chloride, chromium, copper, fluoride, iodine, iron, manganese, molybdenum, selenium and zinc.

Non-limiting examples of macro minerals include calcium, magnesium, potassium and sodium.

The nutritional requirements of these components (exemplified with poultry and piglets/pigs) are listed in Table A of WO 01/58275. Nutritional requirement means that these
25 components should be provided in the diet in the concentrations indicated.

In the alternative, the animal feed additive of the invention comprises at least one of the individual components specified in Table A of WO 01/58275. At least one means either of, one or more of, one, or two, or three, or four and so forth up to all thirteen, or up to all fifteen individual
30 components. More specifically, this at least one individual component is included in the additive of the invention in such an amount as to provide an in-feed-concentration within the range indicated in column four, or column five, or column six of Table A.

In a still further embodiment, the animal feed additive of the invention comprises at least one of the below vitamins, preferably to provide an in-feed-concentration within the ranges
35 specified in the below Table 1 (for piglet diets, and broiler diets, respectively).

Table 1: Typical vitamin recommendations

Vitamin	Piglet diet	Broiler diet
Vitamin A	10,000-15,000 IU/kg feed	8-12,500 IU/kg feed
Vitamin D3	1800-2000 IU/kg feed	3000-5000 IU/kg feed
Vitamin E	60-100 mg/kg feed	150-240 mg/kg feed
Vitamin K3	2-4 mg/kg feed	2-4 mg/kg feed
Vitamin B1	2-4 mg/kg feed	2-3 mg/kg feed
Vitamin B2	6-10 mg/kg feed	7-9 mg/kg feed
Vitamin B6	4-8 mg/kg feed	3-6 mg/kg feed
Vitamin B12	0.03-0.05 mg/kg feed	0.015-0.04 mg/kg feed
Niacin (Vitamin B3)	30-50 mg/kg feed	50-80 mg/kg feed
Pantothenic acid	20-40 mg/kg feed	10-18 mg/kg feed
Folic acid	1-2 mg/kg feed	1-2 mg/kg feed
Biotin	0.15-0.4 mg/kg feed	0.15-0.3 mg/kg feed
Choline chloride	200-400 mg/kg feed	300-600 mg/kg feed

Other feed ingredients

The composition of the invention may further comprise colouring agents, stabilisers, growth improving additives and aroma compounds/flavourings, polyunsaturated fatty acids (PUFAs); reactive oxygen generating species, anti-microbial peptides and anti-fungal polypeptides.

Examples of colouring agents are carotenoids such as beta-carotene, astaxanthin, and lutein.

Examples of aroma compounds/flavourings are creosol, anethol, deca-, undeca-and/or dodeca-lactones, ionones, irone, gingerol, piperidine, propylidene phthalide, butylidene phthalide, capsaicin and tannin.

Examples of antimicrobial peptides (AMP's) are CAP18, Leucocin A, Tritrpticin, Protegrin-1, Thanatin, Defensin, Lactoferrin, Lactoferricin, and Ovispirin such as Novispirin (Robert Lehrer, 2000), Plectasins, and Statins, including the compounds and polypeptides disclosed in WO 03/044049 and WO 03/048148, as well as variants or fragments of the above that retain antimicrobial activity.

Examples of antifungal polypeptides (AFP's) are the *Aspergillus giganteus*, and *Aspergillus niger* peptides, as well as variants and fragments thereof which retain antifungal activity, as disclosed in WO 94/01459 and WO 02/090384.

5 Examples of polyunsaturated fatty acids are C18, C20 and C22 polyunsaturated fatty acids, such as arachidonic acid, docosohexaenoic acid, eicosapentaenoic acid and gamma-linoleic acid.

Examples of reactive oxygen generating species are chemicals such as perborate, persulphate, or percarbonate; and enzymes such as an oxidase, an oxygenase or a syntethase.

10 The composition of the invention may further comprise at least one amino acid. Examples of amino acids which are used in animal feed are lysine, alanine, beta-alanine, threonine, methionine and tryptophan.

Methods of Releasing Galactose

15 In another aspect, the invention relates to a method of releasing galactose from plant based material, comprising treating the plant based material with the animal the animal feed or animal feed additive of the first aspect of the invention, the alpha-galactosidase of the second aspect of the invention, the granule of the third aspect of the invention or the liquid formulation of the fourth aspect of the invention.

20 In one embodiment, the animal feed comprises one or more formulating agents as defined herein. In one embodiment, the animal feed comprises one or more additional enzymes as defined herein. In one embodiment, the animal feed comprises one or more microbes as defined herein. In one embodiment, the animal feed comprises plant based material from the subclass *rosids*. In a preferred embodiment, the animal feed has been pelleted.

25 In an embodiment, the plant based material is soybean, wild soybean, beans, lupin, tepary bean, scarlet runner bean, slimjim bean, lima bean, French bean, Broad bean (fava bean), chickpea, lentil, peanut, Spanish peanut, canola, rapeseed (oilseed rape) or pea or in a processed form such as soybean meal, full fat soy bean meal, soy protein concentrate (SPC), fermented soybean meal (FSBM) or any combination thereof. In a preferred embodiment, the plant based material is soybean or soybean meal.

Methods of Improving Animal Performance

30 In another aspect, the invention relates to a method of improving one or more performance parameters of an animal comprising administering to the animal the animal feed or animal feed additive of the first aspect of the invention, the alpha-galactosidase of the second aspect of the invention, the granule of the third aspect of the invention or the liquid formulation of the fourth aspect of the invention.

35 In a further aspect, the invention relates to a method of improving one or more performance parameters of an animal comprising administering to the animal the animal feed or

animal feed additive of the first aspect of the invention or the alpha-galactosidase of the second aspect of the invention and plant based material, such that the plant based material is administered together or separately with the polypeptide of the invention.

In one embodiment, the term 'improving one or more performance parameters of an animal' means that there is an increase in body weight gain. In another embodiment, the term 'improving one or more performance parameters of an animal' means that there is an improved feed conversion ratio. In a further embodiment, 'the term 'improving one or more performance parameters of an animal' means that there is an increased feed efficiency. In a further embodiment, the term 'improving one or more performance parameters of an animal' means that there is an increase in body weight gain and/or an improved feed conversion ratio and/or an increased feed efficiency.

In one embodiment, the animal feed comprises one or more formulating agents as defined herein. In one embodiment, the animal feed comprises one or more additional enzymes as defined herein. In one embodiment, the animal feed comprises one or more microbes as defined herein. In one embodiment, the animal feed comprises plant based material from the subclass *rosids*. In a preferred embodiment, the animal feed has been pelleted.

In an embodiment, the plant based material is soybean, wild soybean, beans, lupin, tepary bean, scarlet runner bean, slimjim bean, lima bean, French bean, Broad bean (fava bean), chickpea, lentil, peanut, Spanish peanut, canola, rapeseed (oilseed rape) or pea or in a processed form such as soybean meal, full fat soy bean meal, soy protein concentrate (SPC), fermented soybean meal (FSBM) or any combination thereof. In a preferred embodiment, the plant based material is soybean or soybean meal.

Method for improving the nutritional value of animal feed

The term improving the nutritional value of an animal feed means improving the availability of nutrients in the feed. The nutritional values refers in particular to improving the solubilisation and degradation of the raffinose family of oligosaccharides (RFOs), such as the trisaccharide raffinose, the tetrasaccharide stachyose, and the pentasaccharide verbascose, thereby increasing the amount of galactose released which can be utilised by the animal. Consequently, an improved galactose release will result in an improvement of the nutritional value of the feed, thus resulting in increased growth rate and/or weight gain and/or feed conversion (i.e. the weight of ingested feed relative to weight gain).

Thus the invention further relates to a method for improving the nutritional value of an animal feed comprising treating the animal feed with an animal feed or animal feed additive of the first aspect of the invention, the alpha-galactosidase of the second aspect of the invention, the alpha-galactosidase of the second aspect of the invention, the granule of the third aspect of the invention or the liquid formulation of the fourth aspect of the invention. In an embodiment, the animal feed will have improved nutrient digestibility.

In one embodiment, the composition comprises one or more formulating agents as defined herein. In one embodiment, the composition comprises one or more additional enzymes as defined herein. In one embodiment, the composition comprises one or more microbes as defined herein. In a preferred embodiment, the composition is a granule that optionally comprises a salt and/or wax and/or a flour coating.

In one embodiment, the animal feed additive comprises one or more formulating agents as defined herein. In one embodiment, the animal feed additive comprises one or more additional enzymes as defined herein. In one embodiment, the animal feed additive comprises one or more microbes as defined herein. In one embodiment, the animal feed additive comprises one or more vitamins, one or more minerals and/or one or more amino acids. In a preferred embodiment, the animal feed additive is a granule that optionally comprises a salt and/or wax and/or a flour coating.

In an embodiment, the plant based material is from the taxonomic subclass *rosids*. In one aspect, the plant based material is from the taxonomic order *Fabales*, such as the family *Fabaceae*, preferably the subfamilies *Caesalpinioideae* or *Mimosoideae* or *Papilionoideae*, or more preferably from the tribes *Phaseoleae*, *Cicereae*, *Genisteae*, *Fabeae*, *Dalbergieae* or *Phaseoleae*. In one aspect, the plant based material is from the taxonomic order *Brassicales*, such as the family *Brassicaceae*, preferably the tribe *Brassiceae*, more preferably the family *Brassica*.

In particular embodiments, the plant based material is soybean, wild soybean, beans, lupin, tepary bean, scarlet runner bean, slimjim bean, lima bean, French bean, Broad bean (fava bean), chickpea, lentil, peanut, Spanish peanut, canola, rapeseed (oilseed rape) or pea or in a processed form such as soybean meal, full fat soy bean meal, soy protein concentrate (SPC), fermented soybean meal (FSBM) or any combination thereof. In a preferred embodiment, the plant based material is soybean or soybean meal.

In a preferred embodiment, the animal feed has been pelleted. The animal feed may be treated with the enzyme of the invention before the pelleting step or sprayed on after the pelleting step.

Method for reducing the anitnutritional effects of an animal feed

An excessive amount of oligosaccharides in the hindgut can result in antinutritional effects due to flatulence production. By reducing the amount of oligosaccharide fermentation, the antinutritional effects of some animal feeds can be reduced resulting in improved gut and animal health.

Thus the invention further relates to a method for reducing the anitnutritional effects of an animal feed comprising adding to the feed the alpha-galactosidase of the second aspect of the invention, the granule of the third aspect of the invention or the liquid formulation of the fourth aspect of the invention.

In one embodiment, the composition comprises one or more formulating agents as defined herein. In one embodiment, the composition comprises one or more additional enzymes as defined herein. In one embodiment, the composition comprises one or more microbes as defined herein. In a preferred embodiment, the composition is a granule that optionally comprises a salt and/or wax and/or a flour coating.

In one embodiment, the animal feed additive comprises one or more formulating agents as defined herein. In one embodiment, the animal feed additive comprises one or more additional enzymes as defined herein. In one embodiment, the animal feed additive comprises one or more microbes as defined herein. In one embodiment, the animal feed additive comprises one or more vitamins, one or more minerals and/or one or more amino acids. In a preferred embodiment, the animal feed additive is a granule that optionally comprises a salt and/or wax and/or a flour coating.

In an embodiment, the plant based material is from the taxonomic subclass *rosids*. In one aspect, the plant based material is from the taxonomic order *Fabales*, such as the family *Fabaceae*, preferably the subfamilies *Caesalpinioideae* or *Mimosoideae* or *Papilionoideae*, or more preferably from the tribes *Phaseoleae*, *Cicereae*, *Genisteae*, *Fabeae*, *Dalbergieae* or *Phaseoleae*. In one aspect, the plant based material is from the taxonomic order *Brassicales*, such as the family *Brassicaceae*, preferably the tribe *Brassiceae*, more preferably the family *Brassica*.

In particular embodiments, the plant based material is soybean, wild soybean, beans, lupin, tepary bean, scarlet runner bean, slimjim bean, lima bean, French bean, Broad bean (fava bean), chickpea, lentil, peanut, Spanish peanut, canola, rapeseed (oilseed rape) or pea or in a processed form such as soybean meal, full fat soy bean meal, soy protein concentrate (SPC), fermented soybean meal (FSBM) or any combination thereof. In a preferred embodiment, the plant based material is soybean or soybean meal.

In a preferred embodiment, the animal feed has been pelleted. The animal feed may be treated with the enzyme of the invention before the pelleting step or sprayed on after the pelleting step.

Methods of Preparing an Animal Feed

In another aspect, the invention relates to a method of preparing an animal feed, comprising mixing the animal feed additive of the first aspect of the invention, the alpha-galactosidase of the second aspect of the invention, the granule of the third aspect of the invention or the liquid formulation of the fourth aspect of the invention with plant based material.

In one embodiment, the composition comprises one or more formulating agents as defined herein. In one embodiment, the composition comprises one or more additional enzymes as defined herein. In one embodiment, the composition comprises one or more microbes as defined herein. In a preferred embodiment, the composition is a granule that optionally comprises a salt and/or wax and/or a flour coating.

In one embodiment, the animal feed additive comprises one or more formulating agents as defined herein. In one embodiment, the animal feed additive comprises one or more additional enzymes as defined herein. In one embodiment, the animal feed additive comprises one or more microbes as defined herein. In one embodiment, the animal feed additive comprises one or more vitamins, one or more minerals and/or one or more amino acids. In a preferred embodiment, the animal feed additive is a granule that optionally comprises a salt and/or wax and/or a flour coating.

In an embodiment, the plant based material is from the taxonomic subclass *rosids*. In one aspect, the plant based material is from the taxonomic order *Fabales*, such as the family *Fabaceae*, preferably the subfamilies *Caesalpinioideae* or *Mimosoideae* or *Papilionoideae*, or more preferably from the tribes *Phaseoleae*, *Cicereae*, *Genisteae*, *Fabeae*, *Dalbergieae* or *Phaseoleae*. In one aspect, the plant based material is from the taxonomic order *Brassicales*, such as the family *Brassicaceae*, preferably the tribe *Brassiceae*, more preferably the family *Brassica*.

In particular embodiments, the plant based material is soybean, wild soybean, beans, lupin, tepary bean, scarlet runner bean, slimjim bean, lima bean, French bean, Broad bean (fava bean), chickpea, lentil, peanut, Spanish peanut, canola, rapeseed (oilseed rape) or pea or in a processed form such as soybean meal, full fat soy bean meal, soy protein concentrate (SPC), fermented soybean meal (FSBM) or any combination thereof. In a preferred embodiment, the plant based material is soybean or soybean meal.

In a preferred embodiment, the animal feed has been pelleted. The animal feed may be treated with the enzyme of the invention before the pelleting step or sprayed on after the pelleting step.

Uses

The present invention is also directed to methods for using the polypeptides having alpha-galactosidase activity, or compositions thereof, for e.g. animal feed. The present invention is also directed to processes for using the polypeptides having alpha-galactosidase activity, or compositions thereof, such as e.g. those described below.

Use in Animal Feed

The present invention is also directed to methods for using the alpha-galactosidases of the invention in animal feed.

The term animal includes all animals. Examples of animals are non-ruminants, and ruminants. Ruminant animals include, for example, animals such as sheep, goats, and cattle, e.g. beef cattle, cows, and young calves. In a particular embodiment, the animal is a non-ruminant animal. Non-ruminant animals include mono-gastric animals, e.g. pigs or swine (including, but not limited to, piglets, growing pigs, and sows); poultry such as turkeys, ducks and chicken (including but not limited to broiler chicks, layers); horses (including but not limited to hotbloods, coldbloods

and warm bloods), young calves; and fish (including but not limited to salmon, trout, tilapia, catfish and carps; and crustaceans (including but not limited to shrimps and prawns).

In the use according to the invention the alpha-galactosidases can be fed to the animal before, after, or simultaneously with the diet. The latter is preferred.

5 In a particular embodiment, the alpha-galactosidase, in the form in which it is added to the feed, or when being included in a feed additive, is well-defined. Well-defined means that the alpha-galactosidase preparation is at least 50% pure as determined by Size-exclusion chromatography (see Example 12 of WO 01/58275). In other particular embodiments the alpha-galactosidase preparation is at least 60, 70, 80, 85, 88, 90, 92, 94, or at least 95% pure as
10 determined by this method.

A well-defined alpha-galactosidase preparation is advantageous. For instance, it is much easier to dose correctly to the feed a alpha-galactosidase that is essentially free from interfering or contaminating other alpha-galactosidases. The term dose correctly refers in particular to the objective of obtaining consistent and constant results, and the capability of optimizing dosage
15 based upon the desired effect.

For the use in animal feed, however, the alpha-galactosidase need not be that pure; it may e.g. include other enzymes, in which case it could be termed an alpha-galactosidase preparation.

The alpha-galactosidase preparation can be (a) added directly to the feed, or (b) it can be
20 used in the production of one or more intermediate compositions such as feed additives or premixes that is subsequently added to the feed (or used in a treatment process). The degree of purity described above refers to the purity of the original alpha-galactosidase preparation, whether used according to (a) or (b) above.

25 Preferred Embodiments of the Invention

Preferred embodiments of the invention are described in the set of items below.

1. An animal feed additive comprising one or more GH36 polypeptides having alpha-galactosidase activity selected from the group consisting of:
30 (a) a polypeptide having at least 80%, e.g., at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the polypeptide of SEQ ID NO: 3;
(b) a polypeptide encoded by a polynucleotide that hybridizes under high stringency
35 conditions, or very high stringency conditions with
 - (i) the mature polypeptide coding sequence of SEQ ID NO: 1,
 - (ii) the cDNA sequence thereof, or

- (iii) the full-length complementary strand of (i) or (ii);
- (c) a polypeptide encoded by a polynucleotide having at least 80%, e.g., at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%,
5 at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1;
- (d) a variant of the polypeptide selected from the group consisting of SEQ ID NO: 3, wherein the variant has alpha-galactosidase activity and comprises one or more amino acid substitutions, and/or one or more amino acid deletions, and/or
10 one or more amino acid insertions or any combination thereof in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 positions;
- (e) a polypeptide comprising the polypeptide of (a), (b), (c) or (d) and a N-terminal and/or C-terminal His-tag and/or HQ-tag;
15
- (f) a polypeptide comprising the polypeptide of (a), (b), (c) or (d) and a N-terminal and/or C-terminal extension of up to 10 amino acids, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids; and
- (g) a fragment of the polypeptide of (a), (b), (c) or (d) having alpha-galactosidase activity and having at least 90% of the length of the mature polypeptide.
20
2. The animal feed additive of item 1 wherein the polypeptide has improved gastric stability compared to control.
- 25 3. The animal feed additive of item 2 wherein control is defined as SEQ ID NO: 4.
4. The animal feed additive of any of items 2 to 3 wherein gastric stability is measured as the half-life at 40°C pH3 with 0.1 mg/ml pepsin.
- 30 5. The animal feed additive of item 1 wherein the polypeptide has a half-life of at least 90 minutes, such as at least 2 hours, 3 hours or at least 4 hours.
6. The animal feed additive of item 5 wherein half-life is measured at 40°C pH3 with 0.1 mg/ml pepsin.
- 35 7. The animal feed additive of any of items 1 to 6 wherein the polypeptide has increased alpha-galactosidase activity compared to control.

8. The animal feed additive of item 7 wherein control is defined as SEQ ID NO: 4.
9. The animal feed additive of any of items 7 to 8 wherein alpha-galactosidase activity is measured using 4-nitrophenyl α -D-galactopyranoside as substrate.
10. The animal feed additive of any of items 7 to 9 wherein alpha-galactosidase activity is at least 100000 (mOD/min)/(mg/ml), such as at least 150000, at least 200000, at least 250000, or at least 300000 (mOD/min)/(mg/ml).
11. The animal feed additive of any of items 1 to 6, wherein the polypeptide has at least 40%, such as at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 100% of the alpha-galactosidase activity of the polypeptide of SEQ ID NO: 3.
12. The animal feed additive of item 11 wherein alpha-galactosidase activity is measured using 4-nitrophenyl α -D-galactopyranoside as substrate.
13. The animal feed additive of any of items 1 to 12 wherein the polypeptide comprises or consists of amino acids 1 to 721 of SEQ ID NO: 2 or amino acids 1 to 721 of SEQ ID NO: 3.
14. The animal feed additive of any of items 1 to 13 further comprising one or more components selected from the list consisting of:
- one or more vitamins;
 - one or more minerals;
 - one or more amino acids;
 - one or more prebiotics;
 - one or more phytogenics;
 - one or more organic acids; and
 - one or more other feed ingredients.
15. The animal feed additive of any of items 1 to 14 further comprising one or more formulating agents.
16. The animal feed additive of item 15, wherein the one or more formulating agent is selected from the group consisting of glycerol, ethylene glycol, 1, 2-propylene glycol or 1, 3-propylene

glycol, sodium chloride, sodium benzoate, potassium sorbate, sodium sulfate, potassium sulfate, magnesium sulfate, sodium thiosulfate, calcium carbonate, sodium citrate, dextrin, glucose, sucrose, sorbitol, lactose, starch and cellulose or any combination thereof.

- 5 17. The animal feed additive of any of items 1 to 16 further comprising one or more additional enzymes.
18. The animal feed additive of item 17 wherein the one or more additional enzymes is selected from the group consisting of acetylxy lan esterase, acylglycerol lipase, amylase, alpha-
10 amylase, beta-amylase, arabinofuranosidase, cellobiohydrolases, cellulase, feruloyl esterase, galactanase, alpha-galactosidase, beta-galactosidase, beta-glucanase, beta-glucosidase, lysophospholipase, lysozyme, alpha-mannosidase, beta-mannosidase (mannanase), phytase, phospholipase A1, phospholipase A2, phospholipase D, protease, pullulanase, pectinesterase, triacylglycerol lipase, xylanase, beta-xylosidase or any
15 combination thereof.
- 19 The animal feed additive of any of items 1 to 18 further comprising one or more microbes.
20. The animal feed additive of item 19, wherein the one or more microbes is selected from the
20 group consisting of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus pumilus*, *Bacillus polymyxa*, *Bacillus megaterium*, *Bacillus coagulans*, *Bacillus circulans*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, *Bifidobacterium sp.*, *Carnobacterium sp.*, *Clostridium butyricum*, *Clostridium sp.*, *Enterococcus faecium*, *Enterococcus sp.*, *Lactobacillus sp.*, *Lactobacillus acidophilus*,
25 *Lactobacillus farciminus*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactococcus lactis*, *Lactococcus sp.*, *Leuconostoc sp.*, *Megasphaera elsdenii*, *Megasphaera sp.*, *Pediococcus acidilactici*, *Pediococcus sp.*, *Propionibacterium thoenii*, *Propionibacterium sp.* and *Streptococcus sp.* or any combination thereof.
- 30 21. An isolated polypeptide having alpha-galactosidase activity, selected from the group consisting of:
- (a) a polypeptide having at least 96.5%, e.g., at least 97%, at least 97.5%, at least 98%, at least 98.5%, at least 99%, at least 99.5%, or 100% sequence identity to the polypeptide of SEQ ID NO: 3;
- 35 (b) a polypeptide encoded by a polynucleotide having at least 96.5%, e.g., at least 97%, at least 97.5%, at least 98%, at least 98.5%, at least 99%, at least 99.5%,

- or 100% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1;
- 5 (c) a variant of the polypeptide selected from the group consisting of SEQ ID NO: 3, wherein the variant has alpha-galactosidase activity and comprises one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 or 27 positions;
- 10 (d) a polypeptide comprising the polypeptide of (a), (b) or (c) and a N-terminal and/or C-terminal His-tag and/or HQ-tag;
- (e) a polypeptide comprising the polypeptide of (a), (b) or (c) and a N-terminal and/or C-terminal extension of up to 10 amino acids, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids; and
- 15 (f) a fragment of the polypeptide of (a), (b) or (c) having alpha-galactosidase activity and having at least 90% of the length of the mature polypeptide.
22. The polypeptide of item 21, wherein the polypeptide comprises or consists of amino acids amino acids 1 to 721 of SEQ ID NO: 2 or amino acids 1 to 721 of SEQ ID NO: 3.
- 20 23. A composition comprising the polypeptide of any of items 21 to 22.
24. The composition of item 23 further comprising one or more formulating agents.
- 25 25. The composition of item 24, wherein the one or more formulating agent is selected from the group consisting of glycerol, ethylene glycol, 1, 2-propylene glycol or 1, 3-propylene glycol, sodium chloride, sodium benzoate, potassium sorbate, sodium sulfate, potassium sulfate, magnesium sulfate, sodium thiosulfate, calcium carbonate, sodium citrate, dextrin, glucose, sucrose, sorbitol, lactose, starch and cellulose or any combination thereof.
- 30 26. A granule comprising a GH36 polypeptide having having alpha-galactosidase activity, wherein the polypeptide is selected from the group consisting of:
- 35 (a) a polypeptide having at least 80%, e.g., at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the polypeptide of SEQ ID NO: 3;
- (b) a variant of SEQ ID NO: 3, wherein the variant has alpha-galactosidase activity and comprises one or more amino acid substitutions, and/or one or more amino

- acid deletions, and/or one or more amino acid insertions or any combination thereof in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 positions;
- 5 (c) a polypeptide comprising the polypeptide of (a) or (b) and a N-terminal and/or C-terminal His-tag and/or HQ-tag;
- (d) a polypeptide comprising the polypeptide of (a) or (b) and a N-terminal and/or C-terminal extension of up to 10 amino acids, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids; and
- 10 (e) a fragment of the polypeptide of (a) or (b) having alpha-galactosidase activity and having at least 90% of the length of the mature polypeptide.
27. A granule comprising the animal feed additive of any of items 1 to 20, one or more polypeptides of any of items 21 to 22 or the composition of any of items 23 to 25.
- 15 28. The granule of any of items 26 to 27 wherein the granule is coated.
29. The granule of item 28 wherein the coating comprises a salt and/or wax and/or a flour.
- 20 30. A liquid formulation comprising one or more GH36 polypeptides having alpha-galactosidase activity, wherein the liquid formulation comprises:
- (A) 0.001% to 25% w/w of polypeptide having alpha-galactosidase activity wherein the polypeptide having alpha-galactosidase activity is selected from the group consisting of:
- 25 (a) a polypeptide having at least 80%, e.g., at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the polypeptide of SEQ ID NO: 3;
- (b) a variant of SEQ ID NO: 3, wherein the variant has alpha-galactosidase activity and comprises one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 positions;
- 30 (c) a polypeptide comprising the polypeptide of (a) or (b) and a N-terminal and/or C-terminal His-tag and/or HQ-tag;
- 35

- (d) a polypeptide comprising the polypeptide of (a) or (b) and a N-terminal and/or C-terminal extension of up to 10 amino acids, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids; and
- (e) a fragment of the polypeptide of (a) or (b) having alpha-galactosidase activity and having at least 90% of the length of the mature polypeptide; and
- 5 (B) water.
31. The liquid formulation of item 30, wherein the polypeptide having alpha-galactosidase activity is dosed between 0.001% to 25% w/w of liquid formulation, preferably 0.01% to 25% w/w, more preferably 0.05% to 20% w/w, more preferably 0.2% to 15% w/w, even more preferably 0.5% to 15% w/w or most preferably 1.0% to 10% w/w polypeptide.
- 10
32. The liquid formulation of any of items 30 to 31, wherein the formulation further comprises 20% to 80% w/w of polyol.
- 15
33. The liquid formulation of item 32, wherein the polyol is selected from the group consisting of glycerol, sorbitol, propylene glycol (MPG), ethylene glycol, diethylene glycol, triethylene glycol, 1, 2-propylene glycol or 1, 3-propylene glycol, dipropylene glycol, polyethylene glycol (PEG) having an average molecular weight below about 600 and polypropylene glycol (PPG) having an average molecular weight below about 600 or any combination thereof.
- 20
34. The liquid formulation of any of items 30 to 33, wherein the formulation further comprises 0.01% to 2.0% w/w preservative.
- 25
35. The liquid formulation of item 34, wherein the preservative is selected from the group consisting of sodium sorbate, potassium sorbate, sodium benzoate and potassium benzoate or any combination thereof.
36. The liquid formulation of any of items 30 to 35 further comprising one or more components selected from the list consisting of:
- 30
- one or more enzymes;
 - one or more microbes;
 - one or more vitamins;
 - one or more minerals;
 - 35 one or more amino acids;
 - one or more phytochemicals;
 - one or more prebiotics;

one or more organic acids; and
one or more other feed ingredients.

- 5 37. A method of preparing an animal feed comprising applying the liquid formulation of any of items 30 to 36 onto plant based material.
38. The method of item 37, wherein the liquid formulation is applied via a spray.
- 10 39. The method of any of items 37 to 38, wherein the plant based material is selected from the group consisting of soybean, wild soybean, beans, lupin, tepary bean, scarlet runner bean, slimjim bean, lima bean, French bean, Broad bean (fava bean), chickpea, lentil, peanut, Spanish peanut, canola, rapeseed (oilseed rape) or pea or in a processed form such as soybean meal, full fat soy bean meal, soy protein concentrate (SPC), fermented soybean meal (FSBM) or any combination thereof.
- 15 40. The method of any of items 37 to 39, wherein the plant based material is in pelleted form.
41. A pelleted animal feed prepared using the method of any of items 37 to 40.
- 20 42. An animal feed comprising plant based material and the animal feed additive of any of items 1 to 20, one or more polypeptides of any of items 21 to 22, the composition of any of items 23 to 25, the granule of any of items 26 to 29 or the liquid formulation of any of items 30 to 36.
- 25 43. The animal feed of item 42, wherein the plant based material is from the taxonomic subclass *rosids*.
- 30 44. The animal feed of item 42, wherein the plant based material is from the family *Fabaceae*, preferably the sub-family *Papilionoideae*, more preferably from the tribe *Phaseoleae*, *Cicereae*, *Genisteae*, *Fabeae*, *Dalbergieae* or *Phaseoleae* or any combination thereof
- 35 45. The animal feed of item 42, wherein the plant based material is soybean, wild soybean, beans, lupin, tepary bean, scarlet runner bean, slimjim bean, lima bean, French bean, Broad bean (fava bean), chickpea, lentil, peanut, Spanish peanut, canola, rapeseed (oilseed rape) or pea or in a processed form such as soybean meal, full fat soy bean meal, soy protein concentrate (SPC), fermented soybean meal (FSBM) or any combination thereof.

46. A pelleted animal feed comprising plant based material and the animal feed additive of any of items 1 to 20, one or more polypeptides of any of items 21 to 22, the composition of any of items 23 to 25, the granule of any of items 26 to 29 or the liquid formulation of any of items 30 to 36.
- 5
47. The pelleted animal feed of item 46, wherein the plant based material is from the subclass *rosids*, preferably the family *Fabaceae*, more preferably the sub-family *Papilionoideae* or even more preferably is from the tribe *Phaseoleae*, *Cicereae*, *Genisteeae*, *Fabeae*, *Dalbergieae* or *Phaseoleae* or any combination thereof.
- 10
48. The pelleted animal feed of item 46, wherein the plant based material is soybean, wild soybean, beans, lupin, tepary bean, scarlet runner bean, slimjim bean, lima bean, French bean, Broad bean (fava bean), chickpea, lentil, peanut, Spanish peanut, canola, rapeseed (oilseed rape) or pea or in a processed form such as soybean meal, full fat soy bean meal, soy protein concentrate (SPC), fermented soybean meal (FSBM) or any combination thereof.
- 15
49. A method of releasing galactose from plant based material, comprising treating the plant based material with the animal feed additive of any of items 1 to 20, one or more polypeptides of any of items 21 to 22, the composition of any of items 23 to 25, the granule of any of items 26 to 29 or the liquid formulation of any of items 30 to 36.
- 20
50. A method of improving one or more performance parameters of an animal comprising administering to one or more animals the animal feed additive of any of items 1 to 20, one or more polypeptides of any of items 21 to 22, the composition of any of items 23 to 25, the granule of any of items 26 to 29, the liquid formulation of any of items 30 to 36, the animal feed of any of items 42 to 45 or the pelleted animal feed of any of items 41 or 46 to 48.
- 25
51. The method of item 50, wherein the performance parameter is selected from the list consisting of body weight gain (BWG), European Production Efficiency Factor (EPEF) and Feed Conversion Ratio (FCR) or any combination thereof.
- 30
52. A method for improving the nutritional value of an animal feed comprising plant based material, comprising mixing the plant based material with the animal feed additive of any of items 1 to 20, one or more polypeptides of any of items 21 to 22, the composition of any of items 23 to 25, the granule of any of items 26 to 29 or the liquid formulation of any of items 30 to 36.
- 35

53. A method of preparing an animal feed, comprising mixing the animal feed additive of any of items 1 to 20, one or more polypeptides of any of items 21 to 22, the composition of any of items 23 to 25, the granule of any of items 26 to 29 or the liquid formulation of any of items 30 to 36 with plant based material.
54. The methods of any of items 49 to 53, wherein the plant based material is from the taxonomic subclass *rosids*.
55. The methods of any of items 49 to 53, wherein the plant based material is from the family *Fabaceae*, preferably the sub-family *Papilionoideae*, more preferably from the tribe *Phaseoleae*, *Cicereae*, *Genisteae*, *Fabeae*, *Dalbergieae* or *Phaseoleae* or any combination thereof.
56. The methods of any of items 49 to 53, wherein the plant based material is soybean, wild soybean, beans, lupin, tepary bean, scarlet runner bean, slimjim bean, lima bean, French bean, Broad bean (fava bean), chickpea, lentil, peanut, Spanish peanut, canola, rapeseed (oilseed rape) or pea or in a processed form such as soybean meal, full fat soy bean meal, soy protein concentrate (SPC), fermented soybean meal (FSBM) or any combination thereof.
57. Use of the animal feed additive of any of items 1 to 20, one or more polypeptides of any of items 21 to 22, the composition of any of items 23 to 25, the granule of any of items 26 to 29, the liquid formulation of any of items 30 to 36, the animal feed of any of items 42 to 45 or the pelleted animal feed of any of items 41 or 46 to 48:
in animal feed;
in animal feed additives;
in the preparation of a composition for use in animal feed;
for improving the nutritional value of an animal feed;
for increasing digestibility of the animal feed;
for improving one or more performance parameters in an animal; and/or
for releasing galactose from plant based material.
58. A polynucleotide encoding the polypeptide of any of items 21 to 22.

59. A nucleic acid construct or expression vector comprising the polynucleotide of item 58 operably linked to one or more control sequences that direct the production of the polypeptide in an expression host.
- 5 60. A recombinant host cell comprising the polynucleotide of item 58 operably linked to one or more control sequences that direct the production of the polypeptide.
61. A method of producing the polypeptide of any of items 21 to 22, comprising:
- 10 (a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and
- (b) recovering the polypeptide.
62. A method of producing the polypeptide of any of items 21 to 22, comprising:
- 15 (a) cultivating the recombinant host cell of item 60 under conditions conducive for production of the polypeptide; and
- (b) recovering the polypeptide.
63. A transgenic plant, plant part or plant cell transformed with a polynucleotide encoding the polypeptide of any of items 21 to 22.
- 20 64. A whole broth formulation or cell culture composition comprising a polypeptide of any of items 21 to 22.

25 The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

Examples

Strains

30 The alpha-galactosidases were derived from bacterial strains isolated from environmental samples by standard microbiological isolation techniques. Strains were identified and taxonomy was assigned based on DNA sequencing of the 16S ribosomal genes (Table 2).

Table 2: Isolation of fungal strain

Strain	Source	Country	Year	SEQ ID NO of gene	SEQ ID NO of polypeptide
<i>Penicillium pseudopulvillorum</i>	Soil	Nicaragua	1949	1	2

Chromosomal DNA isolated from pure cultures of the individual strains with the DNeasy Blood & Tissue Kit from Qiagen (Hilden, Germany) was subjected to full genome sequencing using
 5 Illumina technology. Genome sequencing, the subsequent assembly of reads and the gene discovery (i.e. annotation of gene functions) is known to the person skilled in the art and the service can be purchased commercially.

The genome sequence was analyzed for alpha-galactosidases from the CAZY database family GH36 (Lombard et al. The Carbohydrate-active enzymes database CAZy. Nucleic Acids
 10 Res 2013, 42:D490–D495.) This analysis identified a gene encoding a putative secreted alpha-galactosidase with the nucleotide sequence given in SEQ ID NO: 1.

Alpha-Galactosidase Assay

Alpha-galactosidase activity can be determined using 4-nitrophenyl α -D-galactopyranoside (product code O-PNPBGAL, available from Megazyme International, Bray, Co.
 15 Wicklow, Ireland) as follows.

The enzyme was diluted using 100 mM MES (Sigma) buffer pH 7.0 ± 0.05 in 2-fold dilutions and then the 4-nitrophenyl α -D-galactopyranoside (1 mg/ml in 100 mM MES buffer pH 7.0 ± 0.05 , prepared immediately before use) was added in the solution containing the enzyme. The respective galactosidase activity was followed directly in the buffer by measuring the
 20 absorbance of released pNP (para-nitro-phenol) at 405 nm for 5 minutes as function of time at room temperature (typically 23°C). A concentration of 1 mg/mL of enzyme is a good starting point; it will however depend from enzyme to enzyme and their specific activity.

The activity is calculated as the slope of a plot of absorbance versus time (units: mOD/min) using the 1 – 5 minute time window and the 0-2 absorbance window. The activity can then be
 25 converted to specific activity by dividing the activity for the concentration of the enzyme (units: (mOD/min)/(mg/ml)).

Galactose Assay

Introduction

The concentration of galactose monosaccharides in a solution was measured
 30 spectrophotometrically after enzymatic hydrolysis of a galactose-rich substrate; soybean meal.

Summarizing, the enzyme(s) were incubated in a 10 w/v% slurry of soybean meal at pH 6.5±0.05 for 2 hours at 40±2 °C. The supernatant was then analyzed in an assay based on the Raffinose/Galactose kit from Megazyme (product name K-RAFGA). First α-D-galactose in the supernatant was converted to β-D-galactose with the enzyme galactose mutarotase. Then β-D-galactose was oxidised by NAD⁺ to D-galactonic acid in the presence of β-galactose dehydrogenase. The amount of NADH formed in this reaction was stoichiometric with the amount of D-Galactose in the supernatant. NADH concentration was then measured by the increase in absorbance at 340nm.

Soybean meal slurry

10 A 10 w/v% slurry of soybean meal was prepared from soybean meal milled to a 0.5 mm particle size and 0.1 M citric acid-phosphate buffer, pH 6.5±0.05.

0.1 M citric acid-phosphate buffer, pH 6.5±0.05 was heated to a temperature of approximately 40 °C while stirring. The preheated buffer was then transferred to the soybean meal. The resulting slurry was stirred while being heated (temperature was not monitored at this point – heating was only applied to ensure that temperature would not decrease too much while the slurry stirred). The slurry was then transferred with a pre-wetted wide-bore pipette to the vessel in which it should be incubated. The slurry was pipetted from an approximately central point in the mix. The time elapsed from the mixing of the slurry until transfer to the last incubation vessel was, at most, 15 minutes. Stirring speed was adjusted in such a way that particles were evenly distributed in the slurry.

Dilution of enzymes

The enzymes were diluted to their desired concentrations in ultrapure water. The concentration to which the enzymes were diluted to was based on the prior concentration of the enzyme in mg enzyme protein per mL (mg EP/mL) and the mass (kg) of dry matter (soybean meal) in each incubation vessel.

$$V_{enzyme}(mL) = \frac{c_{enzyme}(mg \frac{EP}{mL})}{m_{SBM}(kg)}$$

D-(+)-galactose standards

A standard curve was prepared from D-(+)-galactose and ultrapure water. A D-(+)-galactose stock was prepared by dissolving D-(+)-galactose in ultrapure water to a final concentration of 250 mg galactose per mL. The stock solution was diluted in a two-fold dilution row to obtain six standards with concentrations of 250, 125, 62.5, 31.25, 15.625 and 7.813 mg galactose per mL.

Incubation of α -galactosidases on soybean meal

The incubation vessels with the 10 w/v% slurry of soybean meal were heated to a stable temperature of 40 ± 2 °C while stirring. When a stable temperature had been achieved, the six D-(+)-galactose standards were added to the incubation vessels to in-vessel concentrations of 5, 2.5, 1.25, 0.625, 0.313 and 0.157 mg galactose per mL incubation volume. Each standard was incubated in duplicates.

The diluted enzymes were then added to their respective incubation vessels in the volumes required to reach their desired concentrations (in mg EP/kg soybean meal). Each enzyme treatment was incubated in triplicates.

10 Additionally, two times three incubation vessels were included without standards or enzyme treatments as blank treatments to obtain the baseline galactose concentration in the soybean meal slurry.

The incubation vessels were incubated at 40 ± 2 °C, while stirring for 2 hours. After incubation the vessels were centrifuged at 1500 g at 5 °C for 15 minutes.

15 Determination of galactose concentration

The supernatants in the now centrifuged incubation vessels were then analyzed in an assay based on the Raffinose/Galactose kit from Megazyme (product name K-RAFGA). Three reagents from the K-RAFGA kit was used in the assay: Assay Buffer (supplied and ready in Bottle 1 in the kit), β -NAD reagent (supplied in Bottle 2 in the kit, prepared as described in the kit prior to use) and GalDH + GalM solution (supplied in Bottle 3 in the kit, diluted 1:1 in ultrapure water prior to use). All steps described in the following were carried out using an Eppendorf 5075 automated pipetting system.

25 First the supernatants from the centrifuged incubation vessels were diluted 10 times in 0.1 M citric acid-phosphate buffer, pH 6.5 ± 0.05 (1 part supernatant plus 9 parts 0.1 M citric acid-phosphate buffer, pH 6.5 ± 0.05).

30 69 μ L of each diluted supernatant was then transferred to a new vessel and 34 μ L of ultrapure water was added to the diluted supernatants (which will be referred to as assay samples from here on out). Then 69 μ L Assay Buffer was added to the assay samples followed by dilution in 687 μ L ultrapure water. 34 μ L β -NAD reagent was added to the assay samples, followed by addition of 14 μ L GalDH + GalM solution and vigorous mixing.

262 μ L of each assay sample was then transferred to a 96 well micro titer plate. Absorbance in each well of the 96 well micro titer plate was measured at 340 nm at 40 ± 2 °C for a duration of 20 minutes or until absorbance in each well had reached a stable level. When a stable absorbance had been reached this stable absorbance was used in later calculations.

Calculation of galactose concentration

Absorbance of the assay samples from the galactose standards in the incubation vessels were used as a standard curve (6 standards, 5, 2.5, 1.25, 0.625, 0.313 and 0.157 mg galactose per mL incubation volume, n = 2 per standard). An equation for the galactose standard curve was calculated in excel, where y is OD₃₄₀ and x is galactose concentration in mg galactose per mL incubation volume:

$$OD_{340} = a * c_{gal} \left(\frac{mg}{mL} \right) + b$$

Galactose concentration in mg galactose per mL incubation volume for each sample was then given by:

$$c_{gal} \left(\frac{mg}{mL} \right) = \frac{OD_{340} - b}{a}$$

Galactose concentrations were then calculated on a dry-matter basis (g galactose per kg soybean meal) and are reported in the examples below:

$$c_{gal} \left(\frac{g}{kg} SBM \right) = \frac{c_{gal} \left(\frac{mg}{mL} \right) * V_{sample} (mL)}{m_{SBM} (g)}$$

Example 1: Cloning of GH36 alpha-galactosidase (SEQ ID NO: 1)

The alpha-galactosidase with nucleotide sequence SEQ ID NO: 1 and the peptide translation of the protein shown in SEQ ID NO: 2 was PCR amplified from genomic DNA isolated from *Penicillium pseudopulvillum* and cloned into the expression vector pDAu222 as described in WO 2013024021 using BamHI and XhoI restriction sites.

The sequence of the alpha-galactosidase encoding gene cloned in the expression vector was confirmed and the expression construct was transformed into the *Aspergillus oryzae* strain MT3568 (WO 11/057140) to produce the secreted mature peptide with protein sequence SEQ ID NO: 12. Transformants were selected on acetamide during regeneration from protoplasts and subsequently re-isolated under selection (Christensen *et al.*, 1988, *Biotechnology* 6, 1419-1422 and WO 04/032648).

For production of the recombinant alpha-galactosidase, a single *Aspergillus* transformant was cultured in two 500ml baffled flasks each containing 150ml of DAP-4C-1 medium (WO 12/103350). The cultures were shaken on a rotary table at 100 RPM at 30°C for 4 days. The culture broth subsequently was separated from cellular material by passage through a 0.22 um filter.

Example 2: Purification of GH36 alpha-galactosidases (SEQ ID NO: 3)

Filtrated broth was adjusted to pH7.0 and filtrated on 0.22µm PES filter (Nalge Nunc International, Nalgene labware cat#595-4520). Following, the filtrate was added 1.8M ammonium sulphate. The filtrate was loaded onto a Phenyl Sepharose™ 6 Fast Flow column (high sub) (GE Healthcare, Piscataway, NJ, USA) equilibrated with 1.2M ammonium sulphate, 25mM HEPES

pH7.0. After wash with 1.0M ammonium sulphate, the bound proteins were batch eluted with 25 mM HEPES pH 7.0. Fractions were collected and analyzed by SDS-PAGE. The fractions were pooled and applied to a Sephadex™ G-25 (medium) (GE Healthcare, Piscataway, NJ, USA) column equilibrated in 25 mM HEPES pH 7.5. The fractions were applied to a SOURCE™ 15Q (GE Healthcare, Piscataway, NJ, USA) column equilibrated in 25 mM HEPES pH 7 and bound proteins were eluted with 25mM HEPES pH 7, 1 M sodium chloride over ca. 20CV. Fractions were collected and analyzed by SDS-PAGE.

Example 3: Specific activity of the GH36 alpha-galactosidase of SEQ ID NO: 3

The specific activity of the GH36 alpha-galactosidase of the invention (SEQ ID NO: 3) was measured using the Alpha-Galactosidase Assay as disclosed herein and compared to the activity of the prior art GH36 alpha-galactosidase from *Aspergillus niger* (SEQ ID NO: 4). The results are presented in table 3.

Table 3: Specific activity of the GH36 alpha-galactosidases (SEQ ID NO: 3 and 4)

GH36 alpha-galactosidase	Specific Activity on pNP substrate (mOD/min)/(mg/ml)
SEQ ID NO: 3	360000
SEQ ID NO: 4	85000

The GH36 alpha-galactosidase of the invention (SEQ ID NO: 3) is significantly more active in releasing pNP from 4-nitrophenyl α -D-galactopyranoside than the prior art alpha-galactosidase of SEQ ID NO: 4.

Example 4: Gastric stability of the GH36 alpha-galactosidase of SEQ ID NO: 3

Reagents

Pepsin from porcine gastric mucosa (Sigma P7000) diluted in 50 mM glycine buffer pH 3.00 \pm 0.05

Stressing buffer 1: 50 mM glycine pH 3.00 \pm 0.05

Stressing buffer 2: 50 mM glycine pH 3.00 \pm 0.05 + 0.1 mg/mL pepsin

Stressing buffer 3: 100 mM MES pH 7.00 \pm 0.05

Recovery buffer: 100 mM MES pH 7.00 \pm 0.05

Substrate working solution: 1 mg/mL α -pNP diluted in 100 mM MES pH 7.00 \pm 0.05. This working solution is made immediately before use.

Procedure

160 μ L of stressing buffer 1, stressing buffer 2 or stressing buffer 3 was added to the wells of a 96 well PCR plate. The PCR plate was incubated at 40 °C for 10 minutes, then 40 μ L of the

alpha-galactosidase sample to be analyzed was added to the wells containing the stressing buffer. Note that the enzyme was diluted so that the activity in stressing buffer 3 was ~250 mOD/min. After 2, 5, 10, 15, 20, 25 and 30 minutes incubation at 40 °C, 20 µL of the stressed enzymes was added to 80 µL of recovery buffer in the wells of a micro titer plate kept at room temperature. 100 µL of substrate working solution was then added to the wells of the micro titer plate. The solution was mixed for 10 seconds at room temperature and absorption at 405 nm was measured every 30 seconds over 5 minutes.

The activity of the α-galactosidase (V0) was calculated as the slope of the time-dependent absorption curve in the 0.0-2.0 OD absorbance window. In order to evaluate the half-life of the enzyme, the activity (V0) was plotted against the incubation time and the data was fitted to an exponential function of the type: $V0(t)=A e^{-\lambda t}$, where V0(t) is the activity V0 at time t, A = V0(0) is the V0 at time = 0 and λ is the exponential decay constant (also known as decay rate). The half-life is then evaluated as $T_{1/2}=(\ln(2))/\lambda$.

The results are presented in table 4.

Table 4: Specific activity of the GH36 alpha-galactosidases (SEQ ID NO: 3 and 4)

GH36 alpha-galactosidase	T _{1/2} (min)	T _{1/2} (min)
	40C pH3 no pepsin	40C pH3 with 0.1 mg/ml pepsin
SEQ ID NO: 3	Stable	Stable
SEQ ID NO: 4	Stable	77±1

The results show that whilst the prior art alpha-galactanase (SEQ ID NO: 4) is stable at pH3 without any pepsin being present, it decomposes relatively quickly in the presence of 0.1 mg/ml pepsin. In comparison, the alpha-galactanase of the invention (SEQ ID NO: 3) is completely stable both with and without pepsin present.

Example 5: Animal feed and animal feed additives comprising an alpha-galactosidase

Animal Feed Additive

A formulation of an alpha-galactosidase of the invention (e.g. SEQ ID NO: 3) containing 0.01g to 10g enzyme protein is added to the following premix (per kilo of premix):

- 5000000 IE Vitamin A
- 1000000 IE Vitamin D3
- 13333 mg Vitamin E
- 1000 mg Vitamin K3
- 750 mg Vitamin B1
- 2500 mg Vitamin B2

	1500	mg	Vitamin B6
	7666	mcg	Vitamin B12
	12333	mg	Niacin
	33333	mcg	Biotin
5	300	mg	Folic Acid
	3000	mg	Ca-D-Panthenate
	1666	mg	Cu
	16666	mg	Fe
	16666	mg	Zn
10	23333	mg	Mn
	133	mg	Co
	66	mg	I
	66	mg	Se
	5.8	%	Calcium
15	25	%	Sodium

Animal Feed

This is an example of an animal feed (broiler feed) comprising the animal feed additive as described above:

20	62.55 % Maize
	33.8% Soybean meal (50% crude protein)
	1.0% Soybean oil
	0.2% DL-Methionine
	0.22% DCP (dicalcium phosphate)
25	0.76% CaCO ₃ (calcium carbonate)
	0.32% Sand
	0.15% NaCl (sodium chloride)
	1 % of the above Premix

30 The ingredients are mixed, and the feed is pelleted at the desired temperature, e.g. 60, 65, 75, 80, 85, 90 or even 95°C.

Liquid Formulation

35 A liquid formulation of an alpha-galactosidase of the invention (e.g. SEQ ID NO: 3) comprises 0.1% to 10 w/w enzyme protein, 40-60% glycerol, 0.1 to 0.5% sodium benzoate and

water. The liquid formulation is sprayed onto the pelleted animal feed described above (in this case the animal feed additive would not include the alpha-galactosidase of the invention present).

5

The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become
10 apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

Claims

What is claimed is:

1. An animal feed additive comprising one or more GH36 polypeptides having alpha-galactosidase activity selected from the group consisting of:
- 5
- (a) a polypeptide having at least 80%, e.g., at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the polypeptide of SEQ ID NO: 3;
- 10
- (b) a polypeptide encoded by a polynucleotide that hybridizes under high stringency conditions, or very high stringency conditions with
- (i) the mature polypeptide coding sequence of SEQ ID NO: 1,
- (ii) the cDNA sequence thereof, or
- (iii) the full-length complementary strand of (i) or (ii);
- 15
- (c) a polypeptide encoded by a polynucleotide having at least 80%, e.g., at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1;
- 20
- (d) a variant of the polypeptide selected from the group consisting of SEQ ID NO: 3, wherein the variant has alpha-galactosidase activity and comprises one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 positions;
- 25
- (e) a polypeptide comprising the polypeptide of (a), (b), (c) or (d) and a N-terminal and/or C-terminal His-tag and/or HQ-tag;
- (f) a polypeptide comprising the polypeptide of (a), (b), (c) or (d) and a N-terminal and/or C-terminal extension of up to 10 amino acids, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids; and
- 30
- (g) a fragment of the polypeptide of (a), (b), (c) or (d) having alpha-galactosidase activity and having at least 90% of the length of the mature polypeptide.
- 35
2. The animal feed additive of claim 1 wherein the polypeptide has improved gastric stability and/or increased alpha-galactosidase activity using 4-nitrophenyl α -D-galactopyranoside as substrate compared to control.

3. The animal feed additive of any of claims 1 to 2 wherein the polypeptide comprises or consists of amino acids 1 to 721 of SEQ ID NO: 2 or amino acids 1 to 721 of SEQ ID NO: 3.
- 5
4. The animal feed additive of any of claims 1 to 3 further comprising one or more components selected from the list consisting of:
- one or more vitamins;
 - one or more minerals;
 - 10 one or more amino acids;
 - one or more prebiotics;
 - one or more phytochemicals;
 - one or more organic acids; and
 - one or more other feed ingredients.
- 15
5. A granule comprising the animal feed additive of any of claims 1 to 4.
6. A liquid formulation comprising one or more GH36 polypeptides having alpha-galactosidase activity, wherein the liquid formulation comprises:
- 20 (A) 0.001% to 25% w/w of polypeptide having alpha-galactosidase activity wherein the polypeptide having alpha-galactosidase activity is selected from the group consisting of:
- (a) a polypeptide having at least 80%, e.g., at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the polypeptide of SEQ ID NO: 3;
 - 25 (b) a variant of SEQ ID NO: 3, wherein the variant has alpha-galactosidase activity and comprises one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 30 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 positions;
 - (c) a polypeptide comprising the polypeptide of (a) or (b) and a N-terminal and/or C-terminal His-tag and/or HQ-tag;
 - (d) a polypeptide comprising the polypeptide of (a) or (b) and a N-terminal and/or 35 C-terminal extension of up to 10 amino acids, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids; and

- (e) a fragment of the polypeptide of (a) or (b) having alpha-galactosidase activity and having at least 90% of the length of the mature polypeptide; and
- (B) water.
- 5 7. An isolated polypeptide having alpha-galactosidase activity, selected from the group consisting of:
- (a) a polypeptide having at least 96.5%, *e.g.*, at least 97%, at least 97.5%, at least 98%, at least 98.5%, at least 99%, at least 99.5%, or 100% sequence identity to the polypeptide of SEQ ID NO: 3;
- 10 (b) a polypeptide encoded by a polynucleotide having at least 96.5%, *e.g.*, at least 97%, at least 97.5%, at least 98%, at least 98.5%, at least 99%, at least 99.5%, or 100% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1;
- (c) a variant of the polypeptide selected from the group consisting of SEQ ID NO: 3, wherein the variant has alpha-galactosidase activity and comprises one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 or 27 positions;
- 15 (d) a polypeptide comprising the polypeptide of (a), (b) or (c) and a N-terminal and/or C-terminal His-tag and/or HQ-tag;
- (e) a polypeptide comprising the polypeptide of (a), (b) or (c) and a N-terminal and/or C-terminal extension of up to 10 amino acids, *e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids; and
- 20 (f) a fragment of the polypeptide of (a), (b) or (c) having alpha-galactosidase activity and having at least 90% of the length of the mature polypeptide.
- 25
8. The polypeptide of claim 7, wherein the polypeptide comprises or consists of amino acids amino acids 1 to 721 of SEQ ID NO: 2 or amino acids 1 to 721 of SEQ ID NO: 3.
- 30
9. A composition comprising the polypeptide of any of claims 7 to 8.
10. An animal feed comprising plant based material and the animal feed additive of any of claims 1 to 4, the granule of claim 5, the liquid formulation of claim 6, the polypeptide of any of claims 7 to 8 or the composition of claim 9.
- 35

11. A method of releasing galactose from plant based material, comprising treating the plant based material with the animal feed additive of any of claims 1 to 4, the granule of claim 5, the liquid formulation of claim 6, the polypeptide of any of claims 7 to 8 or the composition of claim 9
- 5
12. A method of improving one or more performance parameters of an animal comprising administering to one or more animals the animal feed additive of any of claims 1 to 4, the granule of claim 5, the liquid formulation of claim 6, the polypeptide of any of claims 7 to 8, the composition of claim 9 or the animal feed of claim 10.
- 10
13. A method for improving the nutritional value of an animal feed comprising plant based material, comprising mixing the plant based material with the animal feed additive of any of claims 1 to 4, the granule of claim 5, the liquid formulation of claim 6, the polypeptide of any of claims 7 to 8 or the composition of claim 9.
- 15
14. Use of the animal feed additive of any of claims 1 to 4, the granule of claim 5, the liquid formulation of claim 6, the polypeptide of any of claims 7 to 8, the composition of claim 9 or the animal feed of claim 10:
- in animal feed;
- 20 in animal feed additives;
- in the preparation of a composition for use in animal feed;
- for improving the nutritional value of an animal feed;
- for increasing digestibility of the animal feed;
- for improving one or more performance parameters in an animal; and/or
- 25 for releasing galactose from plant based material.
- 15 A polynucleotide encoding the polypeptide of any of claims 7 to 8.
16. A nucleic acid construct or expression vector comprising the polynucleotide of claim 15 operably linked to one or more control sequences that direct the production of the polypeptide in an expression host.
- 30
17. A recombinant host cell comprising the polynucleotide of claim 15 operably linked to one or more control sequences that direct the production of the polypeptide.
- 35
18. A method of producing the polypeptide of any of claims 7 to 8, comprising:

- (a) cultivating the recombinant host cell of claim 17 under conditions conducive for production of the polypeptide; and
- (b) recovering the polypeptide.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/062652

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23K20/189 C12N9/40
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
A23K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 101 457 208 A (FEED RES INST CAAS [CN]) 17 June 2009 (2009-06-17) page 3 - page 4 claims; sequences	1-18
X	CN 104 805 101 A (FEED RES INST CAAS) 29 July 2015 (2015-07-29) paragraphs [0001], [0003] - [0011] claims; examples; sequences	1-18
A	US 6 197 566 B1 (KNAP INGE HELMER [DK] ET AL) 6 March 2001 (2001-03-06) column 1, line 14 - column 2, line 15 column 9, lines 24-41 claims; examples; sequences	1-18
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Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search 31 July 2017	Date of mailing of the international search report 24/08/2017
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Hartlieb, Ariane
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/062652

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