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(54) Title: NON-ANIMAL BOVINE BETA LACTOGLOBULIN

(57) Abstract: The present disclosure describes a genetically modified microbial host cell expressing one or more heterologous genes encoding a modified beta-lactoglobulin (BLG), thereby producing the said modified BLG, wherein the modified BLG comprises at least one substitution of a non-essential and/or non-branched amino acid with an essential and/or branched amino acid compared to a parent unmodified BLG.

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Non-animal bovine beta lactoglobulin

Technical Field

[0001] The present disclosure describes genetically modified host cell producing beta-lactoglobulin (BLG). Also described herein are gene construct and methods useful for producing such BLG as well as modified bovine BLG having improved nutritional value. Also described herein are composition comprising the BLG and use of the BLG in food and/or feed.

Background

[0002] Recombinantly made beta lactoglobulins (BLG's) and their use as substitute milk protein components are known in the art e.g. from WO2020/219596 or WO2021/168343.

[0003] However, there is a continuous need for finding and developing better ways for the recombinant production of such BLG's and for identifying BLG's with improved properties compared to the naturally available BLG's such as BLG's from cow's milk.

Summary

[0004] Over this background art one objective of this disclosure is to present an improved microbial expression host offering better and more efficient production of BLGs. Another objective is to present a modified bovine BLG which has a higher nutritional value than the natural bovine BLG.

[0005] Accordingly, in an aspect this disclosure describes a genetically modified microbial host cell expressing one or more heterologous genes encoding a modified beta-lactoglobulin (BLG), thereby producing the said modified BLG, wherein the BLG comprises at least one substitution of a non-essential and/or non-branched amino acid with an essential and/or branched amino acid compared to a parent unmodified BLG.

[0006] In another aspect this disclosure describes a modified nucleotide sequence which is at least 50% identical to the BLG encoding gene comprised in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11 12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 167, 168, 170 and/or 171 and encodes a modified BLG having at least one additional essential amino acid compared to a parent BLG.

[0007] In another aspect this disclosure describes a polynucleotide construct comprising a nucleotide sequence which is at least 50% identical to the BLG encoding gene comprised in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11 12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96,

98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 167, 168, 170 and/or 171, operably linked to an inducible promoter which is optionally at least 50 % identical to the promoter comprised in SEQ ID NO: 15.

[0008] In another aspect this disclosure describes a cell culture, comprising the host cells described herein and a growth medium.

[0009] In another aspect this disclosure describes a method for producing a BLG comprising:

a) culturing the cell culture described herein at conditions allowing the cell to produce the modified BLG; and

b) optionally recovering and/or isolating the BLG.

[0010] In another aspect this disclosure describes a fermentation composition comprising the cell culture described herein and/or the BLG comprised in the cell culture.

[0011] In another aspect this disclosure describes a modified bovine BLG polypeptide comprising at least one substitution of a non-essential and/or non-branched amino acid with an essential and/or branched amino acid compared to a parent unmodified BLG.

[0012] In final aspects this disclosure describes a food or feed composition comprising the modified bovine BLG polypeptide described herein and a method of enriching a food or feed composition with essential amino acids comprising mixing one or more food or feed ingredients with the modified BLG.

Description of drawings and figures

[0013] The figures included herein are illustrative and simplified for clarity, and they merely show details which are essential to the understanding of the invention, while other details may have been left out.

[0014] **Figure 1** shows the sequence comparison between the amino acid sequence of BLG manufactured in A. oryzae (AoBLG) with the three major BLG variants present in consumer milk (BLG A, B and C). The amino acid modifications introduced in a BLG B background are highlighted as white text with black background (H and V). The only additional amino acid difference between BLG A, B and C is shown as a grey background (D in BLG A) and is not included in the manufactured AoBLG. The sequences shown in figure 1 is included in SEQ ID NOs: 1, 2, 3 and 4

[0015] **Figure 2** shows the SDS PAGE of culture supernatants of A. oryzae strains cultivated in YPM for 4 days at 30 C. Lane M: Molecular Weight Marker; lane 1: strain TFB-Ao0138; lane 2: TFB-Ao0144; lane 3: strain TFB-Ao0145. All strains contain the same copy number of the AoBLG gene inserted at the same chromosomal location (niaD).

[0016] **Figure 3** shows the SDS PAGE of culture supernatants of BLG strains constructed in control

strain TFB-Ao0094 (derived from TFB-Ao0010 to only integrate one copy) using different codon optimized sequences. Strain names indicate the codon optimized sequence used in each case e.g., strains 186TA and 186TB are strains that contain the AoBLG-D sequence in either 2 (186TA) or 4 copies (186TB, see Table 1).

[0017] **Figure 4** shows the SDS PAGE of culture supernatants of BLG strains constructed in control strain TFB-Ao0094 using different signal peptides, cultivated in duplicate. Lane M: Molecular Weight Marker; Lanes 1 and 2: Plectasin signal peptide; Lanes 3 and 4: Cutinase signal peptide (see WO 2005/121333); Lanes 5 and 6: TL lipase signal peptide (Thermomyces lanuginosus lipase, identical to sequence 4 from patent US8586330); Lanes 7 and 8: SP0034 signal peptide (from an A. niger hypothetical glycoside hydrolase); Lanes 9 and 10: SP0002 signal peptide (from the A. niger mannan endo-1,4-beta-mannosidase).

[0018] **Figure 5** shows the strategy applied for the construction of strains for testing SP efficiency, illustrated for the plectasin signal peptide, (Ple SP). Shortly, Fragment 1 is specific for each SP and contains the upstream flank of the wA gene (Fragment FI-wA). Fragment 2 contains the AoBLG coding sequence. Fragment 3 contains the Tamg terminator and the downstream flank of the wA gene (Fragment FIII-wA). The overlapping regions between Fragment 1 and 2 (Overlap 1) and between Fragment 2 and 3 (Overlap 2) are depicted.

[0019] **Figure 6** shows the SDS PAGE of culture supernatants of BLG strains constructed in strain TFB-Ao0094 using different signal peptides, cultivated in duplicate. Lane M: Molecular Weight Marker; Lanes 1 and 2: strain L-BLG-21 (Ple SP); Lanes 3 and 4: strain L-BLG-23 (Cut SP); Lanes 5 and 6: strain L-BLG-25 (Lip SP); Lanes 7 and 8: strain L-BLG-27 (SP034); Lanes 9 and 10: strain L-BLG-29 (SP002).

[0020] **Figure 7** shows the prtT locus in A. oryzae in strain TFB-Ao0010 and the deletion present in strain TFB-Ao0127 and TFB-Ao0131. The coding region of wild type prtT gene is depicted (prtT grey arrow), together with the sequences used for MAD7 cleavage (PS_AoprtT1 and PS_AoprtT2 white arrows). The deleted region is depicted as a black box ("Deletion"). The position of primers used as donor DNA (P0459A and P0459B) and primers for identification of the deletion (P0457 and P0458) are depicted above.

[0021] **Figure 8** shows a map of plasmid pTFB-0075 used containing the AoBLG expression cassette, including the Ple SP (included in the depicted AoBLG) for the construction of multicopy strains.

[0022] **Figure 9** shows the distribution of essential and non-essential amino acids in BLG B and new BLG variants AoBLG, SuperBLG and LeuMax.

[0023] **Figure 10** shows the sequence alignment of new BLG variants AoBLG, SuperBLG and LeuMax using Muscle (Snapgene) using cow milk BLG B variant as reference. The sequences shown in figure 10 are included in SEQ ID NOs: 2, 4, 59 and 58.

[0024] **Figure 11** shows the branched amino acid content in BLG B and new BLG variants AoBLG, SuperBLG and LeuMax.

[0025] **Figure 12** shows prediction of protein folding for bovine BLG B, AoBLG, SuperBLG and LeuMore 2 using AlphaFold. The graphs depict the probability of having a structure. Values above 90% strongly predict a structured form of the protein. Lower values predict unstructured regions of the protein (e.g. SuperBLG).

[0026] **Figure 13** shows Leucine content of new BLG molecules containing increasing number of Leucine residues LeuMore1 (3 additional Leu) (SEQ ID NO: 60) to LeuMore5 (5 additional Leu) (SEQ ID NO:68) compared to both bovine BLG B and AoBLG.

[0027] **Figure 14** shows SDS PAGE of selected strains expressing the different AoBLG variants. (A) Strains (8) containing several copies of the SuperBLG gene (left), a triplicate cultivation of a single copy AoBLG strain (middle) and eight LeuMax multicopy strains (right); (B) From left to right: Single copy AoBLG strain, single copy LeuMore1 (SEQ ID NO: 60) to LeuMore 5 (SEQ ID NO: 68) strains (2-3 strains per variant).

[0028] **Figure 15** shows protease content in the supernatant from a strain with prtT deletion and a "wild type" strain ("wt") without deletion. The left column shows the number of proteases in a pilot fermentation with wt strain and the two columns to the right are duplicates of the strain with prtT deletion.

[0029] **Figure 16** shows expression of AoBLG in different A. niger strains (Lanes 1-4) and P. pastoris strains (Lanes 5-8). Lanes 9 and 10: Control A. niger and P. pastoris strains, respectively. Lane M: Molecular weight marker

Incorporation by reference

[0030] All publications, patents, and patent applications referred to herein are incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. In the event of a conflict between a term herein and a term in an incorporated reference, the term herein prevails and controls.

Detailed Description

[0031] The technical features and advantages of the invention described herein are readily apparent to a person skilled in the art by the below detailed description of embodiments and examples with reference to the figures and drawings included herein.

Definitions

[0032] The term “BLG” or “parent natural BLG” as used herein refers to native or natural beta lactoglobulin, in general a protein well known in the art e.g. from cow’s milk.

[0033] The term “modified BLG” or “modified bovine BLG” as used herein refers to a non-natural BLG which comprises one or more modifications of a naturally occurring BLG. A modified bovine BLG may for example be a bovine BLG in which one or more amino acids have been deleted or substituted with other amino acids to produce an amino acid sequence or protein configuration distinct from the bovine BLG and not found in nature.

[0034] The term "heterologous" or “recombinant” or “genetically modified” and their grammatical equivalents as used herein interchangeably about nucleotides, polypeptides and cells refers to entities "derived from a different species or cell”. For example, a heterologous or recombinant polynucleotide gene is a gene in a host cell not naturally containing that gene, i.e. the gene is from a different species or cell type than the host cell. A heterologous or recombinant polypeptide is a polypeptide produced in a host cell not naturally containing the polypeptide, i.e. the polypeptide is from a different species or cell type than the host cell. Where the terms as used herein about host cells, they refer to host cells comprising and expressing heterologous or recombinant polynucleotides.

[0035] The term “% identity” is used herein about the relatedness between two amino acid sequences or between two nucleotide sequences usings standard alignment software known in the art, and applying settings as instructed for the software, including gaps, to achieve the maximum percent identity/similarity/homology and, if necessary, considering any conservative substitutions according to the NCIUB rules (hftp://www.chem.qmul.ac.uk/iubmb/misc/naseq.html; NC-IUB, Eur. J. Biochem. (1985)), as part of the sequence identity. 5' or 3' extensions nor insertions (for nucleic acids) or N’ or C’ extensions nor insertions (for polypeptides) usually result in a reduction of identity, similarity or homology using such standard software. For example the “% sequence identity”, as used herein, can be calculated from e.g. two amino acid sequences as follows: The sequences are aligned using Version 9 of the Genetic Computing Groups GAP (global alignment program), using the default BLOSUM62 matrix (see below) with a gap open penalty of -12 (for the first null of a gap) and a gap extension penalty of -4 (for each additional null in the gap). After alignment, percentage identity is calculated by expressing the number of matches as a percentage of the number of amino acids in the reference amino acid sequence using the following BLOSUM62 matrix:

	Ala	Arg	Asn	Asp	Cys	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val
Ala	4																			
Arg	-1	5																		
Asn	-2	0	6																	
Asp	-2	-2	1	6																
Cys	0	-3	-3	-3	9															
Gln	-1	1	0	0	-3	5														
Glu	-1	0	0	2	-4	2	5													
Gly	0	-2	0	-1	-3	-2	-2	6												
His	-2	0	1	-1	-3	0	0	-2	8											
Ile	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
Leu	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
Lys	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
Met	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
Phe	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
Pro	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
Ser	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
Thr	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
Trp	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Tyr	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
Val	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

[0036] "Reference sequence" refers to a defined sequence to which another sequence is compared.

[0037] “Substitution” or “substituted” refers to modification of the polypeptide by replacing one amino acid residue with another, for instance the replacement of a Serine residue with a Glycine or Alanine residue in a polypeptide sequence is an amino acid substitution. When used with reference to a polynucleotide, “substitution” or “substituted” refers to modification of the polynucleotide by replacing one nucleotide with another, for instance the replacement of a cytosine with a thymine in a polynucleotide sequence is a nucleotide substitution.

[0038] The term "expression" includes any step involved in the production of a polypeptide (e.g., encoded enzyme) including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

[0039] The term "expression vector" refers to a DNA molecule, either single- or double stranded, either linear or circular, which comprises a polynucleotide encoding a polypeptide and is operably linked to control sequences that provide for its expression. Expression vectors include expression cassettes for the integration of genes into a host cell as well as plasmids and/or chromosomes comprising such genes.

[0040] The term "host cell" refers to any cell type that is susceptible to transformation, transfection, transduction, or the like with a nucleic acid construct or expression vector comprising a polynucleotide to be expressed in the host cell. Host cell encompasses any progeny of a parent cell including those that are not identical to the parent cell due to mutations that occur during replication.

[0041] The term “nucleotide sequence” or "polynucleotide" are used interchangeably herein to denote a polymer of at least two nucleic acid monomer units or bases (e.g., adenine, cytosine, guanine,

thymine) covalently linked by a phosphodiester bond, regardless of length or base modification.

[0042] The term "polynucleotide construct" refers to a polynucleotide, 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, and which comprises a polynucleotide encoding a polypeptide and one or more control sequences.

[0043] The term "operably linked" refers to a configuration in which a control sequence, such as a promoter, is placed at an appropriate position relative to a coding polynucleotide such that the control sequence directs expression of the coding polynucleotide. More generally, a control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequence. A promoter sequence is "operably linked" to a gene when it is in sufficient proximity to the transcription start site of a gene to regulate transcription of the gene.

[0044] As used herein, "promoter" refers to a sequence of DNA, usually upstream (5') of the coding region of a structural gene, which controls the expression of the coding region by providing recognition and binding sites for RNA polymerase and other factors which may be required for initiation of transcription. The selection of the promoter will depend upon the nucleic acid sequence of interest. A suitable “promoter" is generally one which is capable of supporting the initiation of transcription in a bacterium of the invention, causing the production of an mRNA molecule.

[0045] “Polypeptide" and "protein" are used interchangeably herein to denote a polymer of at least two amino acids covalently linked by an amide bond, regardless of length or post- translational modification (e.g., glycosylation, phosphorylation, lipidation, myristylation, ubiquitination, etc.). Included within this definition are D- and L-amino acids, and mixtures of D- and L-amino acids.

[0046] The term “comprise” and “include” as used throughout the specification and the accompanying items as well as variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. These words are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows.

[0047] The articles "a" and "an" are used herein refer to one or to more than one (i.e. to one or at least one) of the grammatical object of the article. By way of example, "an element" may mean one element or more than one element.

[0048] Terms like “preferably”, “commonly”, “particularly”, and “typically” are not utilized herein to limit the scope of the itemized invention nor to imply that certain features are critical, essential, or even important to the structure or function of the itemed invention. Rather, these terms are merely intended to highlight alternative attractive or additional features that may or may not be utilized in a particular embodiment of the present invention.

[0049] The term “cell culture” as used herein refers to a culture medium comprising a plurality of the host cells described herein. A cell culture may comprise a single strain of host cells or may comprise two or more distinct host cell strains. The culture medium may be any medium that may comprise a recombinant host, e.g., a liquid medium (i.e., a culture broth) or a semi-solid medium, and may comprise additional components, e.g., a carbon source; a nitrogen source; a phosphate source; vitamins; trace elements; salts; amino acids; nucleobases; and the like.

[0050] Term "endogenous" or “native” as used herein refers to a gene or a polypeptide in a host cell which originates from the same host cell.

[0051] The terms "substantially" or "approximately" or “about”, as used herein refers to a reasonable deviation around a value or parameter such that the value or parameter is not significantly changed. These terms of deviation from a value should be construed as including a deviation of the value where the deviation would not negate the meaning of the value deviated from. For example, in relation to a reference numerical value the terms of degree can include a range of values plus or minus 10% from that value. For example, deviation from a value can include a specified value plus or minus a certain percentage from that value, such as plus or minus 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% from the specified value.

[0052] Where a numerical limit or range is stated herein, the endpoints are included. Also, all values and sub ranges within a numerical limit or range are specifically included as if explicitly written out.

[0053] The term “and/or” as used herein is intended to represent an inclusive “or”. The wording X and/or Y is meant to mean both X or Y and X and Y. Further the wording X, Y and/or Z is intended to mean X, Y and Z alone or any combination of X, Y, and Z.

[0054] The term “isolated" as used herein about a compound, refers to any compound, which by means of human intervention, has been put in a form or environment that differs from the form or environment in which it is found in nature. Isolated compounds include but are not limited to compounds of the disclosure for which the ratio of the compounds relative to other constituents with which they are associated in nature is increased or decreased. In an important embodiment the amount of compound is increased relative to other constituents with which the compound is associated in nature. In an embodiment the compound of the disclosure may be isolated into a pure or substantially pure form. In this context a substantially pure compound means that the compound is separated from other extraneous or unwanted material present from the onset of producing the compound or generated in the manufacturing process. Such a substantially pure compound preparation contains less than 10%, such as less than 8%, such as less than 6%, such as less than 5%, such as less than 4%, such as less than 3%, such as less than 2%, such as less than 1 %, such as less than 0.5% by weight of other extraneous or unwanted material usually associated with the compound

when expressed natively or recombinantly. In an embodiment the isolated compound is at least 90% pure, such as at least 91% pure, such as at least 92% pure, such as at least 93% pure, such as at least 94% pure, such as at least 95% pure, such as at least 96% pure, such as at least 97% pure, such as at least 98% pure, such as at least 99% pure, such as at least 99.5% pure, such as 100 % pure by weight.

[0055] The term "deletion" as used herein in the context of polynucleotides and genes refers to the manipulation of a gene so that it is no longer expressed in a host cell.

[0056] The term "disruption" as used herein refers to manipulation of a gene or any of the machinery participating in the expression the gene, so that it is no longer expressed in a host cell.

[0057] The term "attenuation" as used herein refers to manipulation of a gene or any of the machinery participating in the expression the gene, so that it the expression of the gene is reduced as compared to expression without the manipulation.

[0058] All methods described herein can be performed in any suitable order of steps unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[0059] All percentages, ratios and proportions herein are by weight, unless otherwise specified. A weight percent (weight %, also as wt. %) of a component, unless specifically stated to the contrary, is based on the total weight of the composition in which the component is included (e.g., on the total amount of the reaction mixture).

[0060] Unless specifically defined herein, all technical and scientific terms used have the same meaning as commonly understood by a skilled person in the fields of biochemistry, genetics, and microbiology.

[0061] All methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, with suitable methods and materials being described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, shall prevail. Further, the materials, methods, and examples are illustrative only and are not intended to be limiting, unless otherwise specified.

[0062] Practice of the invention described herein employs, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, and recombinant DNA methodologies, which are available to the person skilled in the art. Such techniques are explained fully in the literature. See, for example, Current Protocols in Molecular Biology

(Frederick M. AUSUBEL, 2000, Wiley and son Inc, Library of Congress, USA); Molecular Cloning: A Laboratory Manual, Third Edition, (Sambrook et al, 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Pat. No. 4,683,195; Nucleic Acid Hybridization (B. D. Harries & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the series, Methods In ENZYMOLOGY (J. Abelson and M. Simon, eds.-in-chief, Academic Press, Inc., New York), specifically, Vols.154 and 155 (Wu et al. eds.) and Vol. 185, "Gene Expression Technology" (D. Goeddel, ed.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); and Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Genetically modified microbial host cell

1. As described *supra*, in a first aspect a genetically modified microbial host cell is provided expressing one or more heterologous genes encoding a modified beta-lactoglobulin (BLG), thereby producing the said modified BLG, wherein the modified BLG comprises at least one substitution of a non-essential and/or non-branched amino acid with an essential and/or branched amino acid compared to a parent unmodified BLG.

[0063] In an embodiment the the modified BLG comprises an amino acid sequence which is at least 50%, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the BLG comprised in anyone of SEQ ID NO: 1, 2, 3, 4, 56, 58, 60, 62, 64, 66, 68, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167 and/or 169.

[0064] The one or more heterologous genes encoding the BLG can in some embodiments comprise a nucleotide sequence which is at least 50% identical to anyone of the BLG encoding genes comprised in SEQ ID NO: 2, 4 or 6, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to anyone of the BLG encoding genes comprised in SEQ ID NO: 5, 6, 7, 8, 9, 10, 11

12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 167, 168, 170 and/or 171. In some embodiments the one or more heterologous genes encoding the BLG comprise a nucleotide sequence which is the BLG encoding gene set forth in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11, 12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170 and/or 171.

[0065] In some embodiments the modified BLG expressed by the host cell is modified BLG to have a higher fraction of essential and/or branched amino acids compared to the, optionally natural, parent BLG. Such modified BLGs are more attractive than natural BLGs because they have a higher nutritional value which can be critical when using the BLG e.g. as ingredient in food or feed compositions, for example infant formulas for feeding malnourished infants. In some embodiments the modified BLG has an increased fraction of His, Leu and/or Val compared to the natural parent BLG. A preferred modified BLG is a modified bovine BLG. In useful embodiments the modified BLG comprises one or more moieties taken from bovine BLG A (SEQ ID NO: 1), bovine BLG B (SEQ ID NO: 2) and/or bovine BLG C (SEQ ID NO: 3). Further, the modified BLG can include one or more amino acid substitutions compared to the parent natural BLG, such as substitutions of amino acid in anyone of bovine BLG A, bovine BLG B and bovine BLG C. In some embodiments the modified BLG includes at least two substitutions compared to a natural parent BLG, such as exactly two substitutions. Particularly the modified BLG may comprise at least one substitution of a non-essential and/or non-branched amino acid with an essential and/or branched amino acid, optionally compared to a parent unmodified BLG. More particularly the modified BLG may comprise at least at least 2, such as at least 5, such as at least 10, such as at least 15, such as at least 20, such as at least 25 substitutions of a non-essential and/or non-branched amino acid with an essential and/or branched amino acid, optionally compared to a parent unmodified BLG.

[0066] Additionally or alternatively, the modified BLG may comprise at least at least 1, such as at least 2, such as at least 3, such as at least 4, such as 5, such as at least 6, such as at least 7 substitutions of a non-leucine amino acid with a leucine amino acid.

[0067] In a further embodiment the modified BLG comprises up to 7 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 1, 5, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone SEQ ID NO: 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167 and/or 169.

[0068] In a further embodiment the modified BLG comprises one substitution of a non-leucine amino acid with a leucine amino acid in a position corresponding to anyone of position 1, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone of SEQ ID NO: 75, 77, 79, 81, 83, 85 and/or 87.

[0069] In a further embodiment the modified BLG comprises 2 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 1, 5, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone of SEQ ID NO: 73, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107 and/or 109.

[0070] In a further embodiment the modified BLG comprises 3 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone of SEQ ID NO: 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131 and/or 133.

[0071] In a further embodiment the modified BLG comprises 4 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone of SEQ ID NO: 135, 137, 139, 141, 143, 145 and/or 147.

[0072] In a further embodiment the modified BLG comprises 5 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 1, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG (SEQ ID NO: 149, 151, 153, 155, 157, 159 and/or 161).

[0073] In a further embodiment the modified BLG comprises 6 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone of SEQ ID NO: 163, 165 and/or 167.

[0074] In a further embodiment the modified BLG comprises 7 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to position 21, 96, 108, 120, 139, 150 and 153 of the modified BLG set forth in SEQ ID NO: 169.

[0075] In a further embodiment the modified BLG comprises at least one amino acid substitution compared to anyone of bovine BLG A, bovine BLG B and bovine BLG C, optionally at least two substitutions.

[0076] In a preferred embodiment the BLG is a modified bovine BLG C comprising at least one substitution of Alanine with Valine in a position corresponding to position 118 of the BLG C in SEQ ID NO: 3. In another preferred embodiment the modified BLG is a modified BLG A comprising at least one substitution of Glutamine with Histidine at a position corresponding to position 59 of the BLG A in SEQ ID NO: 1. In particular the modified BLG may contain a Histidine and a Valine at positions corresponding to position 59 and 118 respectively in anyone of the BLG's comprised in SEQ ID NO: 1, 2 or 3. Such a modified BLG can be a BLG comprising an amino acid sequence which is at least 50%

identical to the BLG sequence comprised in SEQ ID NO: 4, 56, 58, 60, 62, 64, 66, 68, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167 and/or 169, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the BLG sequence comprised in SEQ ID NO: 4, 56, 58, 60, 62, 64, 66, 68, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167 and/or 169. In further embodiments the one or more heterologous genes encoding the BLG can comprise a nucleotide sequence which is at least 50% identical to the BLG encoding gene comprised in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11 12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 167, 168, 170 and/or 171, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the BLG encoding gene comprised in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11 12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 167, 168, 170 and/or 171.

[0077] In further embodiments the heterologous gene encoding the BLG in the host cell further comprises a moiety coding for a signal peptide fused to the BLG. This signal peptide and the moiety encoding it may also be heterologous to the host cell and/or to the BLG. In some embodiments the signal peptide comprises an amino acid sequence which is at least 50% identical to the signal peptide comprised in SEQ ID NO: 13, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the signal peptide comprised in SEQ ID NO: 13. In other embodiments the nucleotide sequence of the moiety encoding the signal peptide has a nucleotide sequence which is at least 50% identical to the signal peptide encoding sequence comprised in SEQ ID NO: 14, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the signal

peptide encoding sequence comprised in SEQ ID NO: 14. It has been found that advantageously, the signal peptide is in preferred embodiments derived from a natural defensin including but not limited to fungal defensins. One particularly useful example of such fungal defensins is plectasin, in particular plectasin derived from a strain of the genus of Pseudoplectania, such as Pseudoplectania nigrella.

[0078] In further embodiments the heterologous gene encoding the BLG in the host cell is under control of a promoter, preferably a promoter that is heterologous to host cell and/or to the BLG.

[0079] Such promoters include engineered synthetic promoters. The promoter is preferably an inducible promoter, inducible e.g. by nutrients such as starch, sugars, maltose, glucose and the like. In some embodiments the promoter is an amylase promoter. Examples of such useful promoters are promoters which have a nucleotide sequence which is at least 50% identical to the promoter sequence comprised in SEQ ID NO: 15, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the promoter sequence comprised in SEQ ID NO: 15.

[0080] Production of bulk proteins like modified bovine BLG requires a very efficient expression and secretion in the microbial system of choice (such as fungal, yeast or bacterial host cells) and - at the same time - the lowest possible level of endogenous proteases that enable growth but also increases protein degradation. Host cell improvements were made on e.g., identifying efficient signal peptides to ease secretion, and where possible deleting or downregulating protease genes to increase yields and protein stability. Deletion of protease genes may however severely affect the fitness of the strain. The choices for yield improvement in the art are numerous but not widely applicable as high yield production of heterologous proteins is dependent on many traits starting from the primary amino acid sequence and including secretion, folding, post-translational modifications to mention just a few. Furthermore, a system for multicopy strain construction was used in this work that enabled construction of highly producing strains. Accordingly, in further embodiments the host cell can advantageously be modified by one or more further genetic modifications improving the expression and/or secretion of the BLG selected from

a) attenuation, disruption and/or deletion the expression of one or more genes which are endogenous/native to the host cell; and
b) mutation of one or more genes native to the host cell.

[0081] In some embodiment the attenuated, disrupted and/or deleted native genes encodes a protease capable of digesting BLG or its signal peptide.

[0082] It has been found that the attenuation, disruption and/or deletion of native genes advantageously include genes that encode proteases capable of digesting BLG or its signal peptide,

and thereby weaking the production, secretion and/or folding of the BLG protein. It is known in the art that endogenous proteases may be a challenge for production of recombinant proteins at high titers (Arnau et al., 2020). Therefore, a main approach, to increase protein titers for the host cells described herein, was the inactivation/deletion of protease genes. But endogenous proteases may have an essential function in the cell, making it challenging to obtain loss of function strains. The initial strain (TFB-Ao0010) used for BLG production in particular was engineered to provide a very low protease level using several approaches. The genes encoding the two major secreted proteases, alpA (alkaline protease) and nprA (neutral metalloprotease I) were inactivated. In addition, pepE (aspartic endopeptidase) was inactivated and pepC (encoding a serine protease present in vacuoles and involved in degradation of e.g., unfolded proteins) was downregulated. Thus, in the best performing strains selective protease activities were not present or down regulated. The best performing A. oryzae strain was constructed by deleting alpA, nprA, pepE and down regulating pepC. Additionally or alternatively, the prtT gene in host cells encodes a transcription factor (PrtT) that functions as a general protease regulator. Interestingly, it was found that the expression of the major A. oryzae proteases encoded by alpA and nprA was indeed regulated by PrtT. Moreover, although having inactivated major proteases in the host cell, it was unexpectedly found that deletion of prtT in a production host cell results in a remarkable increase in modified BLG, such as AoBLG, titers when comparing to control strains containing the same copy number of the expression cassette (see Figure 2). Accordingly, proteases that are useful to attenuate, disrupt and/or delete include alpA (alkaline protease), nprA (neutral metalloprotease I), pepE (aspartic endopeptidase), and pepC (serine protease). Suitable alpAs are the alpAs having an amino acid sequence as set forth in SEQ ID NO: 16 or 18, and accordingly in a further embodiment an alpA (alkaline protease) comprised in SEQ ID NO: 16 or 18 is attenuated, disrupted and/or deleted.

[0083] Suitable nprAs are the nprAs having an amino acid sequence as set forth in SEQ ID NO: 20 or 22 and accordingly a nprA (neutral metalloprotease I) comprised in SEQ ID NO: 20 or 22 is attenuated, disrupted and/or deleted.

[0084] Suitable pepEs are pepEs having an amino acid sequence as set forth in SEQ ID NO: 36 or 38, and accordingly a pepE having an amino acid sequence comprised in SEQ ID NO: 36 or 38 is attenuated, disrupted and/or deleted.

[0085] Suitable pepCs are pepCs having an amino acid sequence as set forth in SEQ ID NO: 32 or 34 accordingly a pepC having an amino acid sequence comprised in SEQ ID NO: 32 or 34 is attenuated, disrupted and/or deleted.

[0086] Further protease that are suitably deleted or down regulated is described in WO2015/025055. In some embodiments the native protease is attenuated, disrupted and/or deleted by attenuation,

disruption and/or deletion of a native endogenous protease transcription factor controlling the expression the native proteases in the host cell. Such protease transcription factors include prtT or any analogue or ortholog thereof, that may be present in the host cell. The gene encoding the transcription factor suitably has a nucleotide sequence which is at least 50% identical to the prtT sequence comprised in SEQ ID NO: 24 or 26, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the prtT sequence comprised in SEQ ID NO: 24 or 26.

[0087] The folding and assembly of secreted proteins occurs at the Endoplasmic Reticulum (ER). Physiological stimuli generate stress in the ER through the accumulation of misfolded proteins. Under such circumstances, a signaling network termed the Unfolded Protein Response (UPR) becomes activated to rebalance ER homeostasis. At the initial phase of the UPR, the adaptive response is triggered to relieve this stress. Upon activation, the synthesis of ER resident chaperones occurs to increase the ER folding capacity, whereas the folding load in the ER is decreased through attenuation of the global mRNA translation and increased clearance of misfolded proteins by ER-associated degradation (ERAD; Siwecka et al., 2021). These mechanisms are widely conserved in eukaryotic organisms such as S. cerevisiae and A. oryzae. Accordingly, where the host cell is modified by further genetic modifications by mutation of one or more genes native to the host cell for improving the expression and/or secretion of the BLG, in some embodiments the mutated native genes preferably encode an enzyme involved in activation of a transcription factor regulating the Unfolded Protein Response (UPR) in the host cell. In some embodiments the mutation of the enzyme increases the activation of the UPR transcription factor.

[0088] One such factor is HAC1 (or HAC1p or its ortholog HacA) which is involved in the unfolded protein response (UPR) pathway in eukaryotic cells, particularly in yeast such as Saccharomyces cerevisiae or other fungi. When unfolded proteins accumulate in the ER, the HAC1/HacA mRNA undergoes a unique splicing event that produces an active transcription factor. This splicing is catalyzed by an endonuclease, Ire1 (or Ire1p), which senses the ER stress and activates the UPR signaling cascade. The spliced form of HAC1 is then translated into an active transcription factor that translocates to the nucleus and activates the expression of genes involved in ER protein folding. Accordingly, in a preferred embodiment the transcription factor activated by the mutated enzymes is HAC1. Ire1 (inositol responsive enzyme 1), and its ortholog in A. oryzae IreA, is one such enzyme which is an ER-resident transmembrane kinase and endoribonuclease that activates HAC1. HAC1 is a conserved transcription factor that serves as the master regulator involved in UPR and IreA mediates unconventional intron splicing from HAC1 mRNA, as said, leading to the production of functional HAC1

protein that induces genes encoding ER chaperones and ER associated degradation functions (Zhou et al., 2016).

[0089] UPR is typically associated with high level expression of secreted proteins that may result in high levels of unfolded protein in the ER. Accordingly, in further embodiments the mutated enzyme is an ER-resident transmembrane kinase and/or endoribonuclease, in particular an inositol responsive enzyme. The inositol responsive enzyme is suitably Ire1 or an Ire1 Type enzyme or any of its analogues or orthologues. Preferred mutated enzymes are those having an amino acid sequence which is at least 50% identical to the inositol responsive enzyme comprised in SEQ ID NO: 28 or 30, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the inositol responsive enzyme comprised in SEQ ID NO: 28 or 30. In a specific embodiment the mutated enzyme comprises a mutation at the positions corresponding to position A80 and/or A83 of SEQ ID NO: 28, more particularly the mutations are A80T and/or A83T, such as the enzyme comprised in SEQ ID NO: 28, also denoted as IreA (the Ire1 of A. oryzae). Preferably the mutated inositol responsive enzyme comprised an amino acid sequence as set forth in SEQ ID NO: 30. In special embodiments the mutated inositol responsive enzyme is encoded by a gene having a nucleotide sequence which is at least 50% identical to the inositol responsive enzyme encoding sequence comprised in SEQ ID NO: 29 or 31, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the inositol responsive enzyme encoding sequence comprised in SEQ ID NO: 29 or 31. However, IreA does not always contribute to increased yields. On the contrary, IreA is also involved in the degradation of mRNAs of secreted proteins. Regulated Ire1-dependent decay (RIDD) is a feedback mechanism in which Ire1 cleaves endoplasmic reticulum (ER)-localized mRNAs encoding secretory and membrane proteins in eukaryotic cells under ER stress (Tanaka et al., 2023). Accordingly, it was a surprise that using a mutated IreA and in particular in combination with prtT deletion generated an increased production of BLG in the host cell. As shown in figure 2 the use of a mutated IreA and the deletion of prtT both contributed to higher yield of BLG. In further embodiments the host cell comprises at least 2 copies of the heterologous genes encoding the BLG, to allow for overexpression of this product.

[0090] Also, heterologous genes encoding the BLG are codon optimized for expression in a filamentous fungus, optionally A. oryzae, such as the codon optimized genes set forth in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11, 12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136,

138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 167, 168, 170 and/or 171which have shown particularly useful and effective for the recombinant expression of modified bovine BLG. Further, the host cell may comprise further modifications increasing expression, post-translational modification, secretion and/or folding of the BLG.

[0091] Several codon-optimized gene sequences for AoBLG (see table 1) were investigated all resulting in similar high BLG titers. The codon optimized DNA sequences encoding the AoBLG were used to assess protein titers are shown below. All constructions were introduced in multiple copies in strain TFB-Ao0010. To enable assessment of the expression level of each codon optimized sequence, strains containing identical copy number were obtained and used in cultivation in DWP (see figure 3).

Table 1

Codon optimized sequence (plasmid name)	**DNA sequence**
AoBLG-A (pTFB-75) (SEQ ID NO: 5)	TTGATAGTAACTCAGACCATGAAAGGTCTTGATATTCAGAAGGTTGCCGGCACCTGGTACTCATTG GCTATGGCGGCGTCGGACATTTCTCTCTTGGATGCACAGTCCGCGCCCTTGCGTGTTTACGTGGAG GAACTTAAACCTACGCCAGAAGGCGATTTGGAAATATTGCTTCATAAATGGGAAAACGGCGAATG TGCCCAAAAGAAGATTATCGCTGAGAAAACAAAGATCCCCGCGGTATTCAAAATTGATGCCCTCAA TGAAAACAAGGTATTGGTCCTTGATACGGATTACAAGAAATACCTGCTGTTCTGTATGGAGAATAG CGCAGAGCCAGAACAATCTCTTGTTTGTCAATGCCTGGTAAGGACGCCGGAAGTGGATGACGAAG CCCTTGAGAAATTTGACAAGGCTCTGAAGGCGCTTCCTATGCACATCAGACTGTCTTTCAACCCTAC GCAATTGGAGGAGCAGTGTCACATT
AoBLG-B (pTFB-77) (SEQ ID NO: 6)	TTGATAGTGACGCAAACTATGAAAGGGTTGGACATTCAGAAGGTGGCGGGGACTTGGTATAGTCT CGCCATGGCAGCCTCGGACATTAGCTTGCTGGACGCGCAATCTGCCCCATTGCGTGTCTATGTGGA GGAATTGAAGCCAACTCCCGAGGGGGATCTGGAGATTCTTCTCCACAAATGGGAAAATGGAGAAT GTGCACAAAAGAAGATAATCGCAGAAAAAACCAAAATTCCTGCCGTATTTAAAATTGACGCGCTG AACGAGAACAAAGTATTGGTACTTGACACTGACTATAAGAAGTATCTGCTCTTCTGCATGGAGAAT AGCGCGGAGCCAGAGCAAAGTCTTGTCTGTCAGTGCCTGGTCCGCACACCAGAGGTAGATGATGA AGCACTTGAGAAGTTCGATAAGGCCTTGAAAGCCCTTCCCATGCACATACGACTCAGTTTTAACCC CACACAGCTTGAGGAGCAATGCCATATT
AoBLG-C (pTFB-79) (SEQ ID NO: 7)	CTTATAGTCACACAAACGATGAAGGGGCTGGACATCCAGAAGGTCGCAGGTACCTGGTATTCATT GGCTATGGCGGCATCCGATATATCCCTTCTGGACGCCCAATCGGCCCCACTGAGAGTGTACGTTGA GGAGCTCAAGCCAACCCCGGAAGGAGACCTTGAGATTCTGTTGCATAAGTGGGAGAATGGAGAG TGCGCTCAAAAGAAAATTATAGCGGAGAAGACTAAGATACCGGCGGTTTTCAAGATCGATGCCCT TAATGAAAATAAGGTTCTTGTCCTTGATACGGACTATAAAAAATACCTGTTGTTCTGCATGGAGAA CTCCGCTGAACCAGAGCAATCGCTTGTTTGCCAGTGCTTGGTGAGGACACCAGAAGTAGACGATG AGGCCCTTGAAAAATTTGACAAAGCGCTCAAAGCGCTGCCTATGCACATAAGGTTGAGCTTCAACC CGACACAACTGGAAGAACAATGCCATATA
AoBLG-D (pTFB-186) (SEQ ID NO: 8)	CTCATTGTGACTCAGACTATGAAGGGCTTGGATATCCAGAAAGTGGCAGGCACTTGGTACTCGCTC GCCATGGCAGCATCGGACATTTCGTTGCTCGATGCACAGTCCGCACCTTTGCGCGTCTATGTGGAG GAGTTGAAGCCCACACCCGAAGGCGATTTGGAAATCCTCTTGCATAAGTGGGAGAACGGAGAGT GTGCACAGAAAAAGATCATCGCGGAAAAGACCAAGATTCCGGCAGTCTTCAAGATTGACGCCCTC AACGAGAACAAAGTGTTGGTCCTCGACACCGACTACAAAAAGTACCTCCTCTTCTGTATGGAAAAC TCCGCAGAGCCCGAGCAGTCGCTCGTGTGTCAGTGTTTGGTGCGAACACCCGAGGTCGACGACGA GGCCTTGGAGAAATTCGACAAGGCGCTCAAGGCCCTCCCCATGCACATTAGGCTCTCGTTCAACCC GACTCAGTTGGAAGAGCAGTGTCATATC

Codon optimized sequence (plasmid name)	DNA sequence
AoBLG-E (pTFB-188) (SEQ ID NO: 9)	TTGATCGTCACTCAGACTATGAAGGGCCTCGACATCCAGAAAGTGGCAGGTACCTGGTATTCCTTG GCAATGGCAGCGTCGGACATTTCCCTCTTGGACGCCCAGTCCGCACCTCTCCGCGTCTATGTGGAA GAGTTGAAGCCCACACCTGAAGGAGATTTGGAGATCCTCTTGCACAAATGGGAAAACGGCGAGT GTGCCCAGAAGAAAATCATCGCGGAAAAGACCAAGATTCCTGCGGTCTTCAAGATTGATGCGCTC AACGAAAACAAGGTGCTCGTGTTGGACACCGACTACAAAAAGTACCTCCTCTTCTGTATGGAAAAC TCGGCAGAACCCGAACAGTCCCTCGTGTGTCAGTGTCTCGTGAGGACACCCGAAGTGGATGACGA GGCCCTCGAAAAGTTCGATAAGGCACTCAAGGCCTTGCCGATGCACATCCGCCTCTCGTTCAACCC TACTCAGTTGGAGGAACAGTGTCATATC
AoBLG-F (pTFB-217 (SEQ ID NO: 10)	TTGATCGTTACCCAGACCATGAAGGGCTTGGACATCCAGAAGGTTGCCGGTACCTGGTATTCTCTG GCCATGGCCGCTTCTGACATCTCTTTGTTGGACGCCCAGTCTGCTCCACTTCGCGTTTACGTTGAGG AGCTTAAGCCAACCCCAGAGGGTGATCTGGAGATCTTGTTGCACAAGTGGGAGAATGGCGAGTG TGCCCAGAAGAAGATCATCGCTGAGAAGACCAAGATCCCTGCTGTTTTCAAGATCGACGCTCTTAA CGAGAATAAGGTGCTGGTGCTGGACACCGATTACAAGAAGTATTTGTTGTTCTGTATGGAGAACT CTGCTGAGCCAGAGCAGTCTTTGGTTTGCCAGTGCCTTGTTCGCACCCCTGAGGTGGACGACGAG GCTCTTGAGAAGTTCGATAAGGCTTTGAAGGCTCTGCCAATGCACATCCGCTTGAGCTTCAATCCT ACCCAGTTGGAGGAGCAGTGTCACATCTGA
AoBLG-G (pTFB-219) (SEQ ID NO: 11)	CTTATCGTTACCCAGACCATGAAGGGCTTGGATATCCAGAAGGTGGCTGGTACCTGGTACTCTCTT GCCATGGCTGCCTCTGACATCTCTCTTCTTGACGCCCAGTCCGCCCCTCTTCGTGTGTATGTTGAAG AATTGAAGCCGACCCCTGAAGGTGATCTTGAGATCCTGTTGCATAAGTGGGAAAATGGTGAATGT GCCCAGAAGAAGATCATCGCTGAAAAGACCAAGATCCCTGCCGTTTTCAAGATCGACGCTTTGAAT GAAAATAAGGTTCTTGTTTTGGATACCGATTATAAGAAGTACCTGCTGTTCTGCATGGAGAATAGC GCCGAACCCGAACAGAGCCTGGTGTGCCAGTGCCTTGTTCGTACCCCGGAAGTTGACGATGAGGC TTTGGAGAAGTTCGATAAGGCCCTGAAGGCCTTGCCTATGCACATCCGTTTGTCCTTCAATCCTACC CAGCTTGAGGAACAGTGCCACATCTGA
AoBLG-H (pTFB-221) (SEQ ID NO: 12)	TTGATCGTGACCCAGACTATGAAGGGATTGGATATCCAGAAGGTGGCTGGAACCTGGTACTCCTT GGCTATGGCCGCTAGCGACATCTCTCTGTTGGACGCCCAGTCCGCCCCTTTGCGCGTGTACGTTGA GGAACTGAAGCCTACCCCTGAGGGTGATTTGGAAATCTTGCTTCACAAGTGGGAAAACGGTGAAT GCGCTCAAAAGAAGATCATCGCTGAAAAGACCAAGATCCCGGCCGTTTTCAAGATCGACGCCTTG AACGAAAACAAGGTTTTGGTTCTGGATACTGATTACAAGAAGTACTTGCTGTTCTGTATGGAAAAT TCTGCCGAACCGGAACAGTCTCTGGTTTGTCAGTGCCTGGTTCGGACTCCCGAAGTTGATGACGAG GCCCTTGAGAAGTTCGACAAGGCCCTTAAGGCCCTGCCCATGCACATCCGTCTGTCTTTCAACCCTA CTCAACTGGAGGAGCAGTGCCATATCTGA

[0092] The host cell is preferably a eukaryotic, a prokaryotic or an algal cell. Among eukaryotic host cells, fungal cells, optionally a filamentous fungus or yeast are particularly useful and productive when it comes to production of BLG. Particularly, the filamentous fungus, such as those from the genus of Aspergillus, Trichoderma, or Rhizopus are preferred. Such filamentous fungi include the species of Aspergillus sp, Aspergillus oryzae, Aspergillus niger, Trichoderma sp, or Rhizopus sp. Also yeast, e.g. of the genus of Pichia, Saccharomyces, Yarrowia, or Hansenula, can advantageously be used as host cell, including particularly yeast from the species of Pichia pastoris, Pichia sp., Saccharomyces cerevisiae, Yarrowia sp., Yarrowia lipolytica, Hansenula polymorpha, or Hansenula sp.

[0093] More specifically described herein is a genetically modified A. oryzae host cells expressing a modified BLG comprising an amino acid sequence as comprised in or set forth in SEQ ID NO: 4, 56, 58, 60, 62, 64, 66, 68, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153,

155, 157, 159, 161, 163, 165, 167 and/or 169, having a higher fraction of essential and/or branched amino acids compared to a parent, optionally natural, BLG, said host cell expressing one or more codon optimized heterologous genes as comprised in or set forth in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11 12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 167, 168, 170 and/or 171, encoding the modified BLG, wherein the host cell is further modified by attenuating, disrupting or deleting one or more native genes encoding a protease selected from the group of alpA (alkaline protease), nprA (neutral metalloprotease I), pepE (aspartic endopeptidase), and pepC (serine protease), and wherein the host cell is further modified by mutating a native inositol responsive enzyme having two mutations corresponding to positions A80T and A83T as set forth in SEQ ID NO: 28 thereby increasing activation of transcription factor HAC1 in the host cell.

Nucleotides

[0094] As described supra, in a further aspect a modified nucleotide sequence is provided which is at least 50% identical to the BLG encoding gene comprised in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11 12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 167, 168, 170 and/or 171, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the BLG encoding gene comprised in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11 12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 167, 168, 170 and/or 171and encodes a modified BLG having at least one additional essential amino acid compared to a parent BLG. Further a polynucleotide construct is provided which comprises the nucleotide sequence which is at least 50% identical to the BLG encoding gene comprised in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11 12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 167, 168, 170 and/or 171, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the BLG

encoding gene comprised in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11 12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 167, 168, 170 and/or 171. The BLG encoding gene preferably also encodes a signal peptide fused to the BLG. The signal peptide is preferably heterologous to host cell and/or to the BLG. Several signal peptides of fungal origin were compared to identify the optimal sequence for secretion of BLG. No secretion was detected using the native bovine signal peptide (not shown). As shown (see figure 4), the use of an engineered fungal signal peptide derived from plectasin resulted in the highest amount of BLG present in the culture supernatant in a strain containing a single copy of the AoBLG gene (AoBLG-A), while when using a cutinase SP, titers comparable to the use of plectasin SP were observed in single copy strains. Lower titers were obtained for the remaining SP tested. Accordingly, in some embodiments the encoded signal peptide comprises an amino acid sequence which is at least 50% identical to the signal peptide comprised in SEQ ID NO: 13, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the signal peptide comprised in SEQ ID NO: 13. The polynucleotide moiety encoding the signal peptide may be at least 50% identical to the signal peptide encoding sequence comprised in SEQ ID NO: 14, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the signal peptide encoding sequence comprised in SEQ ID NO: 14. In other embodiments the signal peptide is derived from a natural defensin. Natural defensins are known in the art and include for example fungal defensin. One such fungal defensin signal peptide which has shown particularly useful for this use is the plectasin signal peptide, in particular the signal peptide from the plectasin from the genus of Pseudoplectania, such as Pseudoplectania nigrella.

[0095] The gene encoding the BLG and optionally the signal peptide is preferably operably linked to a promoter. The said promoter can be native or heterologous to the host cell and/or to the BLG. In preferred embodiments the promoter is heterologous to both the host cell and to the BLG for optimal performance. In some embodiments the promoter is an engineered synthetic promoter, in other embodiments the promoter is an inducible promoter for example by nutrients such as starch, sugars, maltose, glucose and the like. In still other embodiments, the promoter is an amylase promoter. The nucleotide sequence of the promoter is preferably at least 50% identical to the promoter sequence comprised in SEQ ID NO: 15, such as at least 55%, such as at least 60%, such as at least 65%, such as

at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the promoter comprised in SEQ ID NO: 15. The said polynucleotide construct is preferably comprised in an expression vector, and/or is in itself and expression vector. Furthermore, the host cell described herein also preferably comprises the aforesaid polynucleotide construct.

Cell culture and method of culturing

[0096] As described supra, in a further aspect a cell culture is provided which comprises the host cell described herein and a growth medium, as well as methods are provided for producing BLG by culturing the cell culture at conditions allowing the host cell to produce the BLG; and optionally recovering and/or isolating the BLG. Suitable growth mediums for host cell described herein are well known in the art. The cell culture can be cultivated in a nutrient medium using methods known in the art at conditions suitable for production of the BLG and/or for propagating cell count. For example, the culture 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 fermenters in a suitable medium and under conditions allowing the host cells to grow and/or propagate, optionally to be recovered and/or isolated.

[0097] The cultivation can take place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. The medium will usually contain all nutrients necessary for the growth and survival of the respective host cell, such as carbon and nitrogen sources and other inorganic salts. Suitable media, e.g. minimal or complex media, are available from commercial suppliers, or may be prepared according to published receipts, e.g. the American Type Culture Collection (ATCC) Catalogue of strains. Non-limiting standard medium well known to the skilled person include Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth, MS broth, Yeast Peptone Dextrose, BMMY, GMMY, or Yeast Malt Extract (YM) broth, which are all commercially available. The selection of an appropriate medium may be based on the choice of host cell and/or based on the regulatory requirements for the host cell. The medium may, if desired, contain additional components favoring the host cells over other potentially contaminating microorganisms. Accordingly, in an embodiment a suitable nutrient medium comprises a carbon source (e.g. C6 sugars (such as glucose), maltose, molasses, starch, cellulose, xylan, pectin, lignocellolytic biomass hydrolysate, C5 sugars (such as arabinose or xylose), acetate, glycerol, plant oils, sucrose, yeast extract, peptone, casamino acids or mixtures thereof), a nitrogen source (e.g. ammonia, ammonium sulphate, ammonium nitrate, ammonium chloride, etc.), an organic nitrogen source (e.g. amines yeast extract, malt extract, peptone, soybean-hydrolysate, or digested fermentative microorganisms etc.)

and inorganic nutrient sources (e.g. phosphate, magnesium, potassium, zinc, iron, etc.). In particular embodiments the medium also contains an inducer inducing expression of the BLG in the host cell.

[0098] Culturing of the host cell may be performed over a period of about 0.5 to about 30 days. The cultivation process may be a batch process, continuous or fed-batch process, suitably performed at a temperature in the range of 0-100 °C or 10-80 °C, for example, from about 20°C to about 50 °C and/or at a pH, for example, from about 2 to about 10. Preferred fermentation conditions for fungal host cells are a temperature in the range of from about 25 °C to about 55 °C and at a pH of from about 3 to about 9. The appropriate conditions are usually selected based on the choice of host cell. Accordingly, in an embodiment the method may further comprise one or more elements/steps selected from:

a) culturing the cell culture in a nutrient medium;
b) culturing the cell culture under aerobic or anaerobic conditions;
c) culturing the cell culture under agitation;
d) culturing the cell culture at a temperature of between 25 to 50 °C;
e) culturing the cell culture at a pH of between 3-9; and
f) culturing the cell culture for between 10 hours to 30 days.

[0099] In some embodiments the method further comprises feeding the cell culture with one or more precursors or substrates or inducers leading to the expression of the BLG. The method can further comprise recovering the BLG and mixing it with one or more carriers, agents, additives, adjuvants and/or excipients. The cell culture and/or the BLG comprised in the cell culture and/or the medium om the cell culture may also be recovered and/or isolated using methods known in the art. For example, the BLG may be recovered or purified from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, precipitation, spray-drying, or lyophilization. In a particular embodiment the method includes a recovery and/or isolation step comprising separating a liquid phase of the cell or cell culture from a solid phase of the cell or cell culture to obtain a supernatant comprising the BLG and/or subjecting the supernatant to one or more steps selected from:

a) separating the supernatant from the solid phase of the cell culture, such as by filtration or gravity separation;
b) contacting the supernatant with one or more adsorbent resins to obtain at least a portion of the produced BLG;
c) contacting the supernatant with one or more ion exchange or reversed-phase chromatography columns in order to obtain at least a portion of the BLG;
d) extracting the BLG; and/or
e) precipitating the BLG by crystallization or evaporating the solvent of the liquid phase; and

optionally isolating the BLG by filtration or gravity separation;

thereby recovering and/or isolating the BLG.

Fermentation compositions

[0100] In a further aspect a fermentation composition is provided which comprises the BLG obtained from the culturing of the cell culture. In some embodiments a majority of the solid cellular material/debris has been separated, such as at least 50%, such as at least 75%, such as at least 95%, such as at least 99% of solid cellular material has been separated from the composition. The fermentation composition may further comprise one or more further compounds or metabolites from the cell culture. Such compounds and/or metabolites of the cell culture include precursors for the BLG as well as compounds selected from trace metals, vitamins, salts, yeast nitrogen base, carbon source, YNB, and/or amino acids of the fermentation. In particular the composition comprises a concentration of the BLG of at least 1 mg/kg composition, such as at least 5 mg/kg, such as at least 10 mg/kg, such as at least 20 mg/kg, such as at least 50 mg/kg, such as at least 100 mg/kg, such as at least 500 mg/kg, such as at least 1.000 mg/kg, such as at least 5.000 mg/kg, such as at least 10.000 mg/kg, such as at least 50.000 mg/kg. The composition may also further comprise one or more carriers, agents, additives and/or excipients.

Modified BLG polypeptides and compositions

[0101] As described supra, in a further aspect a modified BLG polypeptide, optionally a modified bovine BLG polypeptides, such as AoBLG, is provided comprising at least one substitution of a non-essential and/or non-branched amino acid with an essential and/or branched amino acid compared to a parent unmodified BLG. In some embodiments the modified BLG polypeptide comprises at least at least 2, such as at least 5, such as at least 10, such as at least 15, such as at least 20, such as at least 25 substitutions of a non-essential and/or non-branched amino acid with an essential and/or branched amino acid. In some embodiments the modified BLG polypeptide comprises at least 1, such as at least 2, such as at least 3, such as at least 4, such as 5, such as at least 6, such as at least 7 substitutions of a non-leucine amino acid with a leucine amino acid. In some embodiments the modified BLG comprises an amino acid sequence which is at least 50%, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to the BLG comprised in anyone of SEQ ID NO: 1, 2, 3, 4, 56, 58, 60, 62, 64, 66, 68, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145,

147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167 and/or 169. In some embodiments the modified BLG comprises an amino acid sequence as set forth in SEQ ID NO: 4, 56, 58, 60, 62, 64, 66, 68, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167 and/or 169. In some embodiments the modified BLG comprises up to 7 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 1, 5, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone SEQ ID NO: 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167 and/or 169. In some embodiments the modified BLG comprises one substitution of a non-leucine amino acid with a leucine amino acid in a position corresponding to anyone of position 1, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone of SEQ ID NO: 75, 77, 79, 81, 83, 85 and/or 87. In some embodiments the modified BLG comprises 2 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 1, 5, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone of SEQ ID NO: 73, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107 and/or 109. In some embodiments the modified BLG comprises 3 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone of SEQ ID NO: 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131 and/or 133. In some embodiments the modified BLG comprises 4 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone of SEQ ID NO: 135, 137, 139, 141, 143, 145 and/or 147. In some embodiments the modified BLG comprises 5 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 1, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG as set forth in SEQ ID NO: 149, 151, 153, 155, 157, 159 and/or 161. In some embodiments the modified BLG comprises 6 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG as set forth in anyone of SEQ ID NO: 163, 165 and/or 167. In some embodiments the modified BLG comprises 7 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to position 21, 96, 108, 120, 139, 150 and 153 of the modified BLG set forth in SEQ ID NO: 169. In some embodiments the modified BLG comprises at least one amino acid substitution compared to anyone of bovine BLG A, bovine BLG B and bovine BLG C, optionally at least two substitutions. In some embodiments the modified BLG is a modified bovine BLG C comprising at least one substitution of Alanine with Valine in a position corresponding to position 118 of the BLG C in SEQ ID NO: 3. In other embodiments the modified BLG is a modified BLG A comprising at least one

substitution of Glutamine with Histidine at a position corresponding to position 59 of the BLG A in SEQ ID NO: 1. In some embodiments the modified BLG may contain a Histidine and a Valine at positions corresponding to position 59 and 118 respectively in anyone of the BLG's comprised in SEQ ID NO: 1, 2 or 3.

[0102] The BLG is or can be a combination of the three major BLG variants present in cow milk (A, B and C) in a single molecule, incorporating the best possible composition of essential amino acids without introducing any non-natural modification i.e., two amino acid substitutions are introduced in the bovine BLG B backbone, one from BLG A and one from BLG C. Contrary to all other developed recombinant BLGs that are based solely on BLG B, the BLG described herein is therefore nutritionally superior to known recombinant BLG products, while still being representative of/equivalent to drinking cow's milk than a single variant. The amino acid sequence of the AoBLG has a His at position 59 and Val at position 118 replacing Gln59 and Ala118, respectively (see figure 1). These modifications were selected to achieve a combination of not altering natural BLG sequences but combining them in a way that enhances nutritional value – using essential amino acids (His, Leu and/or Val) instead of non-essential amino acid (Gln and Ala). In this way, the AoBLG product has a higher level of essential amino acids than BLG A, B or C in a single molecule, and has a more equivalent sequence compared to the major BLG variants present in milk. The modified BLG polypeptide is also suitably incorporated and/or mixed into a food or feed composition, optionally thereby enriching the food or feed composition with essential amino acids from the modified BLG.

[0103] Having generally described the inventions contained herein, a further understanding can be obtained by reference to specific examples, which are provided below for purposes of illustration only, not intended to be limiting unless otherwise specified.

Sequence listings

[0104] The present application contains a listing of sequences included in the below table A submitted electronically in ST26 format which is hereby incorporated by reference in its entirety.

Table A

SEQ ID NO: 1 -> protein sequence of BLG A
SEQ ID NO: 2 -> protein sequence of BLG B
SEQ ID NO: 3 -> protein sequence of BLG C
SEQ ID NO: 4 -> protein sequence of AoBLG
SEQ ID NO: 5 -> DNA sequence encoding AoBLG-A
SEQ ID NO: 6 -> DNA sequence encoding AoBLG-B
SEQ ID NO: 7 -> DNA sequence encoding AoBLG-C
SEQ ID NO: 8 -> DNA sequence encoding AoBLG-D
SEQ ID NO: 9 -> DNA sequence encoding AoBLG-E

SEQ ID NO: 10 -> DNA sequence encoding AoBLG-F
SEQ ID NO: 11 -> DNA sequence encoding AoBLG-G
SEQ ID NO: 12 -> DNA sequence encoding AoBLG-H
SEQ ID NO: 13 -> protein sequence of Plectasin Signal
SEQ ID NO: 14 -> DNA sequence of Plectasin Signal
SEQ ID NO: 15 -> DNA sequence of PNA2 promoter
SEQ ID NO: 16 -> protein sequence of AlpA protease WILD TYPE
SEQ ID NO: 17 -> DNA sequence of AlpA protease WILD TYPE
SEQ ID NO: 18 -> protein sequence of AlpA protease AFTER DELETION
SEQ ID NO: 19 -> DNA sequence of AlpA protease AFTER DELETION
SEQ ID NO: 20 -> protein sequence of nprA protease WILD TYPE
SEQ ID NO: 21 -> DNA sequence of nprA protease WILD TYPE
SEQ ID NO: 22 -> protein sequence of nprA protease AFTER DELETION
SEQ ID NO: 23 -> DNA sequence of nprA protease AFTER DELETION
SEQ ID NO: 24 -> protein sequence of prtT transcription factor WILD TYPE
SEQ ID NO: 25 -> DNA sequence of prtT transcription factor WILD TYPE
SEQ ID NO: 26 -> protein sequence of prtT transcription factor AFTER DELETION
SEQ ID NO: 27 -> DNA sequence of prtT transcription factor AFTER DELETION
SEQ ID NO: 28 -> protein sequence of IreA WILD TYPE
SEQ ID NO: 29 -> DNA sequence of IreA WILD TYPE
SEQ ID NO: 30 -> protein sequence of IreA MUTANT (A80T/A83T)
SEQ ID NO: 31 -> DNA sequence of IreA MUTANT (A80T/A83T)
SEQ ID NO: 32 -> protein sequence of pepC WILD TYPE
SEQ ID NO: 33 -> DNA sequence of pepC WILD TYPE (own promoter DNA in green)
SEQ ID NO: 34 -> protein sequence of pepC AFTER PROMOTER EXCHANGE
SEQ ID NO: 35 -> DNA sequence of pepC AFTER PROMOTER EXCHANGE
SEQ ID NO: 36 -> protein sequence of pepE protease WILD TYPE
SEQ ID NO: 37 -> DNA sequence of pepE protease WILD TYPE
SEQ ID NO: 38 -> protein sequence of pepE AFTER DELETION
SEQ ID NO: 39 -> DNA sequence of pepE AFTER DELETION
SEQ ID NO: 40 -> DNA sequence of Brick-007
SEQ ID NO: 41 -> DNA sequence of Fragment FI-wA
SEQ ID NO: 42 -> DNA sequence of Fragment FII-wA
SEQ ID NO: 43 -> DNA sequence of Fragment FIII-wA
SEQ ID NO: 44 -> DNA sequence of Ple SP
SEQ ID NO: 45 -> DNA sequence of Cut SP
SEQ ID NO: 46 -> DNA sequence of Lip SP
SEQ ID NO: 47 -> DNA sequence of SP002
SEQ ID NO: 48 -> DNA sequence of SP034
SEQ ID NO: 49 -> DNA sequence of P0200
SEQ ID NO: 50 -> DNA sequence of P0201
SEQ ID NO: 51 -> DNA sequence of P0202
SEQ ID NO: 52 -> DNA sequence of P0203
SEQ ID NO: 53 -> DNA sequence of P0459
SEQ ID NO: 54 -> DNA sequence of P0460
SEQ ID NO: 55 -> DNA sequence of P0461

SEQ ID NO: 56 -> Protein sequence of SuperBLG
SEQ ID NO: 57 -> DNA sequence of SuperBLG
SEQ ID NO: 58 -> Protein sequence of LeuMax
SEQ ID NO: 59 -> DNA sequence of LeuMax
SEQ ID NO: 60 -> LeuMore 1 Protein (Mature sequence, no signal peptide – identical as for AoBLG)
SEQ ID NO: 61 -> LeuMore 1 DNA (no signal peptide or start ATG – identical as for AoBLG)
SEQ ID NO: 62 -> LeuMore 2 Protein (Mature sequence, no signal peptide – identical as for AoBLG)
SEQ ID NO: 63 -> LeuMore 2 DNA (no signal peptide or start ATG – identical as for AoBLG)
SEQ ID NO: 64 -> LeuMore 3 Protein (Mature sequence, no signal peptide – identical as for AoBLG)
SEQ ID NO: 65 -> LeuMore 3 DNA (no signal peptide or start ATG – identical as for AoBLG)
SEQ ID NO: 66 -> LeuMore 4 Protein (Mature sequence, no signal peptide – identical as for AoBLG)
SEQ ID NO: 67 -> LeuMore 4 DNA (no signal peptide or start ATG – identical as for AoBLG)
SEQ ID NO: 68 -> LeuMore 5 Protein (Mature sequence, no signal peptide – identical as for AoBLG)
SEQ ID NO: 69 -> LeuMore 5 DNA (no signal peptide or start ATG – identical as for AoBLG)
SEQ ID NO: 70 -> DNA sequence of BLG-Leu1
SEQ ID NO: 71 -> protein sequence of BLG-Leu1 -> Mutation L1Q
SEQ ID NO: 72-> DNA sequence of BLG-Leu2
SEQ ID NO: 73-> protein sequence of BLG-Leu2 -> Mutation L1Q, Q5L
SEQ ID NO: 74 -> DNA sequence of BLG-Leu3
SEQ ID NO: 75 -> protein sequence of BLG-Leu3 -> Mutation S150L
SEQ ID NO: 76 -> DNA sequence of BLG-Leu4
SEQ ID NO: 77 -> protein sequence of BLG-Leu4 -> Mutation S21L
SEQ ID NO: 78-> DNA sequence of BLG-Leu5
SEQ ID NO: 79 -> protein sequence of BLG-Leu5 -> Mutation A139L
SEQ ID NO: 80 -> DNA sequence of BLG-Leu6
SEQ ID NO: 81 -> protein sequence of BLG-Leu6 -> Mutation P153L
SEQ ID NO: 82-> DNA sequence of BLG-Leu7
SEQ ID NO: 83 -> protein sequence of BLG-Leu7 -> Mutation D96L
SEQ ID NO: 84 -> DNA sequence of BLG-Leu8
SEQ ID NO: 85 -> protein sequence of BLG-Leu8 -> Mutation Q120L
SEQ ID NO: 86-> DNA sequence of BLG-Leu9
SEQ ID NO: 87 -> protein sequence of BLG-Leu9 -> Mutation E108L
SEQ ID NO: 88-> DNA sequence of BLG-Leu10
SEQ ID NO: 89 -> protein sequence of BLG-Leu10 -> Mutation S150L, S21L

SEQ ID NO: 90-> DNA sequence of BLG-Leu11
SEQ ID NO: 91 -> protein sequence of BLG-Leu11 -> Mutation S150L, A139L
SEQ ID NO: 92-> DNA sequence of BLG-Leu12
SEQ ID NO: 93 -> protein sequence of BLG-Leu12 -> Mutation S150L, P153L
SEQ ID NO: 94-> DNA sequence of BLG-Leu13
SEQ ID NO: 95 -> protein sequence of BLG-Leu13 -> Mutation S150L, D96L
SEQ ID NO: 96-> DNA sequence of BLG-Leu14
SEQ ID NO: 97 -> protein sequence of BLG-Leu14 -> Mutation S150L, Q120L
SEQ ID NO: 98-> DNA sequence of BLG-Leu15
SEQ ID NO: 99 -> protein sequence of BLG-Leu15 -> Mutation S150L, E108L
SEQ ID NO: 100 -> DNA sequence of BLG-Leu16
SEQ ID NO: 101-> protein sequence of BLG-Leu16 -> Mutation S21L, Q120L
SEQ ID NO: 102 -> DNA sequence of BLG-Leu17
SEQ ID NO: 103 -> protein sequence of BLG-Leu17 -> Mutation D96L, A139L
SEQ ID NO: 104-> DNA sequence of BLG-Leu18
SEQ ID NO: 105-> protein sequence of BLG-Leu18 -> Mutation D96L, Q120L
SEQ ID NO: 106 -> DNA sequence of BLG-Leu19
SEQ ID NO: 107 -> protein sequence of BLG-Leu19 -> Mutation D96L, E108L
SEQ ID NO: 108 -> DNA sequence of BLG-Leu20
SEQ ID NO: 109 -> protein sequence of BLG-Leu20 -> Mutation Q120L, E108L
SEQ ID NO: 110-> DNA sequence of BLG-Leu21
SEQ ID NO: 111 -> protein sequence of BLG-Leu21 -> Mutation S150L, S21L, A139L
SEQ ID NO: 112-> DNA sequence of BLG-Leu22
SEQ ID NO: 113 -> protein sequence of BLG-Leu22 -> Mutation S150L, S21L, P153L
SEQ ID NO: 114-> DNA sequence of BLG-Leu23
SEQ ID NO: 115-> protein sequence of BLG-Leu23 -> Mutation S150L, A139, P153L
SEQ ID NO: 116-> DNA sequence of BLG-Leu24
SEQ ID NO: 117-> protein sequence of BLG-Leu24 -> Mutation S21L, A139L, P153L
SEQ ID NO: 118-> DNA sequence of BLG-Leu25
SEQ ID NO: 119 -> protein sequence of BLG-Leu25 -> Mutation S150L, S21L, Q120L
SEQ ID NO: 120-> DNA sequence of BLG-Leu26
SEQ ID NO: 121 -> protein sequence of BLG-Leu26 -> Mutation S150L, S21L, D96L
SEQ ID NO: 122 -> DNA sequence of BLG-Leu27

SEQ ID NO: 123 -> protein sequence of BLG-Leu27 -> Mutation S150L, S21L, E108L
SEQ ID NO: 124-> DNA sequence of BLG-Leu28
SEQ ID NO: 125 -> protein sequence of BLG-Leu28 -> Mutation S150L, D96L, Q120L
SEQ ID NO: 126-> DNA sequence of BLG-Leu29
SEQ ID NO: 127 -> protein sequence of BLG-Leu29 -> Mutation S150L, E108L, D96L
SEQ ID NO: 128-> DNA sequence of BLG-Leu30
SEQ ID NO: 129 -> protein sequence of BLG-Leu30 -> Mutation S150L, E108L, Q120L
SEQ ID NO: 130-> DNA sequence of BLG-Leu31
SEQ ID NO: 131 -> protein sequence of BLG-Leu31 -> Mutation S21L, D96L, Q120L
SEQ ID NO: 132-> DNA sequence of BLG-Leu32
SEQ ID NO: 133 -> protein sequence of BLG-Leu32 -> Mutation S21L, E108L, Q120L
SEQ ID NO: 134-> DNA sequence of BLG-Leu33
SEQ ID NO: 135 -> protein sequence of BLG-Leu33 -> Mutation S150L, S21L, A139L, P153L
SEQ ID NO: 136-> DNA sequence of BLG-Leu34
SEQ ID NO: 137 -> protein sequence of BLG-Leu34 -> Mutation S150L, S21L, D96L, Q120L
SEQ ID NO: 138 -> DNA sequence of BLG-Leu35
SEQ ID NO: 139 -> protein sequence of BLG-Leu35 -> Mutation S150L, S21L, D96L, E108L
SEQ ID NO: 140-> DNA sequence of BLG-Leu36
SEQ ID NO: 141 -> protein sequence of BLG-Leu36 -> Mutation S150L, D96L, Q120L, A139L
SEQ ID NO: 142-> DNA sequence of BLG-Leu37
SEQ ID NO: 143 -> protein sequence of BLG-Leu37 -> Mutation S150L, S21L, E108L, Q120L
SEQ ID NO: 144 -> DNA sequence of BLG-Leu38
SEQ ID NO: 145 -> protein sequence of BLG-Leu38 -> Mutation S150L, D96L, Q120L, P153L
SEQ ID NO: 146 -> DNA sequence of BLG-Leu39
SEQ ID NO: 147 -> protein sequence of BLG-Leu39 -> Mutation S21L, D96L, E108L, Q120L
SEQ ID NO: 148-> DNA sequence of BLG-Leu40
SEQ ID NO: 149 -> protein sequence of BLG-Leu40 -> Mutation S150L, S21L, A139L, P153L, D96L
SEQ ID NO: 150-> DNA sequence of BLG-Leu41
SEQ ID NO: 151 -> protein sequence of BLG-Leu41 -> Mutation S150L, S21L, D96L, E108L, Q120L
SEQ ID NO: 152-> DNA sequence of BLG-Leu42
SEQ ID NO: 153 -> protein sequence of BLG-Leu42 -> Mutation S21L, A139L, P153L, D96L, E108L
SEQ ID NO: 154-> DNA sequence of BLG-Leu43
SEQ ID NO: 155 -> protein sequence of BLG-Leu43 -> Mutation S21L, A139L, D96L, E108L, Q120L

SEQ ID NO: 156-> DNA sequence of BLG-Leu44
SEQ ID NO: 157 -> protein sequence of BLG-Leu44 -> Mutation S150L, S21L, A139L, D96L, Q120L
SEQ ID NO: 158-> DNA sequence of BLG-Leu45
SEQ ID NO: 159-> protein sequence of BLG-Leu45 -> Mutation S150L, S21L, A139L, D96L, E108L
SEQ ID NO: 160-> DNA sequence of BLG-Leu46
SEQ ID NO: 161 -> protein sequence of BLG-Leu46 -> Mutation S150L, A139L, P153L, D96L, Q120L
SEQ ID NO: 162 -> DNA sequence of BLG-Leu47
SEQ ID NO: 163 -> protein sequence of BLG-Leu47 -> Mutation S150L, S21L, A139L, D96L, Q120L, E108L
SEQ ID NO: 164 -> DNA sequence of BLG-Leu48
SEQ ID NO: 165 -> protein sequence of BLG-Leu48 -> Mutation S150L, S21L, P153L, D96L, Q120L, E108L
SEQ ID NO: 166-> DNA sequence of BLG-Leu49
SEQ ID NO: 167 -> protein sequence of BLG-Leu49 -> Mutation S150L, S21L, A139L, P153L, D96L, Q120L
SEQ ID NO: 168-> DNA sequence of BLG-Leu50
SEQ ID NO: 169 -> protein sequence of BLG-Leu50 -> Mutation S150L, S21L, A139L, P153L, D96L, Q120L, E108L
SEQ ID NO:170 -> DNA sequence of Pichia pastoris optimised BLG AoBLG (no signal peptide or start ATG)
SEQ ID NO:171 -> DNA sequence of Aspergillus niger optimised BLG AoBLG (no signal peptide or start ATG)

* * *

Examples

Materials and methods

[0105] Chemicals used in the examples herein, e.g. for buffers and substrates, are commercial products of at least reagent grade.

[0106] General used methods of PCR, cloning, etc. were well-known in the art and can for example be found in in " Molecular cloning: A laboratory manual ", Sambrook et al. (1989), Cold Spring Harbor lab., Cold Spring Harbor, N.Y.; Ausubel, F.M. et al. (eds.); " Current protocols in Molecular Biology ", John Wiley and Sons, (1995); Harwood , C. R. , and Cutting, S. M. (eds.). Copy number determination was performed using a QIAcuity digital PCR system, according to the manufacturer's recommendations (Qiagen).

Strain Cultivation

[0107] Deep Well Plates (DWP) containing 3 mL YPM medium (2 g/L yeast extract, 2 g/L peptone, 2 % (w/v) maltose) were inoculated with spores from a transformant strain and incubated at 30° C, 200 rpm for 4 days. To select candidates, biological replicates and duplicate cultivation was performed. This cultivation protocol enables the prediction of yields obtained in bioreactor.

Sucrose Medium

[0108] 1M Sucrose, 0.18 µM $Na_2B_4O_7$, 2.3 µM $CuSO_4$, 4.7 µM $FeSO_4$, 4.7 µM $MnSO_4$, 3.6 µM $NaMoO_4$, 45 µM $ZnSO_4$, 7 mM KCl, 4.3 mM $MgSO_4$, 11.2 mM KH_2PO_4. Transformation plates contained sucrose medium, supplemented with 10 mM $NaNO_3$ and 2 % agar.

Strain Construction

[0109] Strain construction was carried out by transformation of Aspergillus oryzae essentially as described in Christensen et al. (1988); *High-level expression of recombinant genes in Aspergillus oryzae. Biotechnology*, vol. 6, pp. 1419-1422. A. oryzae was grown in a rich nutrient broth. The biomass was separated from the broth by filtration. The enzyme preparation Glucanex® (Novozymes) was added to the biomass in osmotically stabilizing buffer such as 1.2 M $MgSO_4$ buffered to pH 5.0 with sodium phosphate. The suspension was incubated for 60 minutes at 37° C. with gentle agitation to produce protoplasts. Protoplasts were filtered through miracloth to remove mycelial debris. The protoplasts were harvested and washed twice with STC (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5). Protoplasts were finally resuspended in 200-1000 µL STC.

[0110] For transformation, 2-10 µg of plasmid DNA was added to 100 µL protoplast suspension and then 200 µL PEG solution (60 % PEG 4000, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5) was added and the

mixture is incubated for 20 minutes at room temperature. Protoplasts were harvested and washed twice with 1.2 M sorbitol. Protoplasts were finally resuspended in 200 µL 1.2 M sorbitol. Transformants were selected for its ability to grow on minimal sucrose plates using nitrate ($NaNO_3$) as the sole nitrogen source. The transforming DNA either in circular or linear form is targeted to a truncated niaD gene that is restored upon correct integration, as described in WO 2013/178674. After 5-7 days of growth at 37° C, transformants appeared as vigorously growing and sporulating colonies. Transformants were purified through conidiation. For some of the strain modifications (e.g., to introduce the IreA mutation), the CRISPR MAD7 nuclease was used as described elsewhere using one or two guide RNAs and a donor DNA fragment encoding the intended sequence modification, if required (Vanegas et al., 2022).

Strains

[0111] The original recipient strain used in this work, TFB-Ao0010, was derived from A. oryzae BECh2, described in WO2000/39322. The modifications introduced in the strain included 1) reduction of protease levels (e.g., alkaline protease AlpA, Neutral protease NprA among others); 2) deletion of genes encoding major secreted endogenous proteins (including neutral amylase) and 3) inactivation of a gene (ligD) involved in the non-homologous end-joining (NHEJ) pathway – to increase the frequency of homologous recombination and facilitate gene editing (Nakamura et al., –017).

[0112] Using TFB-Ao0010 multicopy strains were obtained that contained different copy numbers of the expression cassette integrated at the niaD locus using the methods described in WO2013/178674. The recipient strain used for comparison of e.g., signal peptide efficiency, TFB-Ao0094, was a further modification that resulted in strains having the expression cassette integrated in single copy at the same niaD locus. The list of strains used is shown in Table 2.

Strain Name	Recipient Strain	Copy no. (AoBLG)	Comment/Property
TFB-Ao0010	-	-	Multicopy recipient strain
TFB-Ao0127	TFB-Ao0010	-	$prtT^-$
TFB-Ao0131	TFB-Ao0127	-	$prtT^-$, IreA
TFB-Ao0138	TFB-Ao0010	5	Multicopy AoBLG in wt prtT, IreA background
TFB-Ao0144	TFB-Ao0127	5	Multicopy AoBLG in $prtT^-$ background
TFB-Ao0145	TFB-Ao0131	5	Multicopy AoBLG in $prtT^-$, IreA mutation background
TFB-Ao0094	TFB-Ao0010	-	Single copy recipient strain
L-BLG-21	TFB-Ao0094	1	Single copy AoBLG, Plectasin SP
L-BLG-23	TFB-Ao0094	1	Single copy AoBLG, Cutinase SP
L-BLG-25	TFB-Ao0094	1	Single copy AoBLG, Lipolase SP
L-BLG-27	TFB-Ao0094	1	Single copy AoBLG, SP034 SP
L-BLG-29	TFB-Ao0094	1	Single copy AoBLG, SP002 SP

Table 2. Used strains of A. oryzae.

Cloning

[0113] For multicopy strain construction, plasmid pTFB-0075 was constructed using NEBuilder® HiFi DNA assembly following the manufacturer's protocol. The backbone plasmid was a shuttle E. coli/Aspergillus vector containing a complementing niaD region (3' niaD), the A. oryzae pyrG as selection marker, an engineered promoter (Pna2) of the Aspergillus niger neutral amylase gene 2 and the Tamg terminator of the glucoamylase gene as described in WO2013/178674). The plasmid contained two adjacent SwaI restriction sites located between the Pna2 promoter and the Tamg terminator. The insert (Brick-0007) encoding the expression cassette for AoBLG and 20/21 bp overlapping DNA flanks (to the backbone plasmid) was obtained as a gBlock from IDT and cloned into the SwaI-digested backbone plasmid.

[0114] For single copy strain construction, co-transformation with three overlapping PCR fragments was performed. Two of the fragments included the upstream and downstream flanking region of the target locus and the third one included AoBLG (Figure 5, see Example 2). As above, sequence overlap between the fragments enables in vivo ligation/insertion at the target locus (Figure 5).

Plasmids

[0115] Plasmid pTFB-0075 is described in Example 3 below (Figure 8)

Protein yield analysis

[0116] SDS-PAGE precast gels (NuPAGE Bis-Tris 4-12%) were used run and stained with Coomassie blue as recommended by the manufacturer (Invitrogen).

Example 1 – Designing an improved modified bovine beta-lactoglobulin (BLG) protein sequence to be produced in Aspergillus oryzae.

[0117] A modified bovine BLG protein sequence was designed (AoBLG) (SEQ ID NO: 4) which was a combination of the three major BLG variants present in cow milk (A, B and C) in a single molecule. The AoBLG amino acid sequence incorporates the best possible composition of essential amino acid without introducing any non-natural modification i.e., two amino acid substitutions were made in the bovine BLG B "backbone", one from BLG A and one from BLG C. Contrary to all other developed recombinant BLGs that are based solely on BLG B, AoBLG is therefore nutritionally superior to current recombinant BLG products. Further, ingestion/consumption of AoBLG is more representative of or

equivalent to intake of cow's milk than a single variant, including the BLG B variant typically chosen for recombinant production.

[0118] The amino acid sequence of the designed modified bovine BLG manufactured in A. oryzae (AoBLG) had a His at position 59 and Val at position 118 replacing Gln59 and Ala118, respectively (see Figure 1). As mentioned above, the rationale for making these changes is a combination of not altering natural BLG sequences but combining them in a way that enhances nutritional value – using essential amino acids (His, LEu and/or Val) instead of non-essential amino acids (Gln and Ala). In this way, the AoBLG product had a higher level of essential amino acids than BLG A, B or C in a single molecule, and had a more equivalent sequence compared to the major BLG variants present in milk. This modified BLG could be produced in commercially feasible levels in microbial cell factories and shown in the following examples.

Example 2 - Signal peptide sequences for increased AoBLG yield.

[0119] The purpose of this experiment was to screen for signal peptides to investigate and identify SPs providing the best yield of AoBLG when expressed in host cells. The investigated SPs are described below (Table 2) and include heterologous SP's Including Ple SP, Cut SP and Lip SP of different length and amino acid composition (Table 3).

Signal peptide	Origin
Plectasin (Ple SP)	Engineered SP derived from the Pseudoplectania nigrella plectasin gene
Cutinase (Cut SP)	Humicola insolens cutinase gene (WO 2005/121333)
Lipase (Lip SP)	Thermomyces lanuginosus lipase gene (US8586330)
SP002	A. niger gene putative glycoside hydrolase
SP034	A. niger gene encoding mannan endo-1,4-beta-mannosidase

Table 3. Origin of the used SP sequences for AoBLG yield.

[0120] To enable comparison of BLG yields using different SP, three overlapping DNA fragments were co-transformed into strain TFB-Ao0094, for each SP. Fragment 1 encodes the upstream region of the A. oryzae colony color gene wA, the Pna2 promoter derived from the A. niger neutral amylase gene and the specific SP. Fragment 2 encodes the mature AoBLG. Fragment 3 encodes the Tamg terminator derived from the A. niger glucoamylase gene and the downstream region of wA (Figure 5).

[0121] In all cases, correct integration of the AoBLG cassette could be monitored by the change from green to white colonies that resulted from disruption of the wA gene. Sequence confirmation was obtained for all strains. Cultivation single copy AoBLG strains was performed in Deep Well Plates (DWP) for 4 days at 30° C and samples from the culture supernatant were used to run an SDS-PAGE.

As shown (Figure 6), the use of the Ple SP derived from plectasin resulted in the highest amount of BLG present in the culture supernatant in a strain (L-BLG-21) containing a single copy of the AoBLG gene inserted at the wA gene (Figure 6). Using a cutinase SP (Cut SP, described in WO 2005/121333, Table 2), AoBLG levels comparable to the use of Ple SP were observed in single copy strain L-BLG-23. Lower titers were obtained for the remaining three SP tested (strains L-BLG-25, L-BLG-27 and L-BLG-29), including a frequently used lipase SP (Lip SP, Table 3, Figure 6). Thus, the use of Ple SP or Cut SP resulted in the highest AoBLG yields.

Example 3 - A combination of modifications of the host strain that result in higher BLG yield in multicopy strains.

[0122] The effects on BLG yield of deletion of the protease regulator prtT (Punt et al., 2008) and the modification of the Inositol responsive, endoplasmic reticulum located ribonuclease/kinase (IreA) was investigated.

PrtT deletion

[0123] Strain TFB-Ao0010 was co-transformed using two MAD7 plasmids as described (Vanegas et al., 2023) in which two protospacer sequences were cloned (P0460 or P0461) and a donor DNA fragment spanned the flanks of the deleted prtT gene (P0459). The expected deletion of the prtT gene including most of the coding region (Figure 7) was confirmed in strain TFB-Ao0127.

[0124] Initially it was believed that the main part of proteases would be removed by deletion of pitT and that deletion of prtT would harm the prtT deleted A. oryzae strain. However, surprisingly, although >25 % of the proteases found in the fermentation broth of a prtT deletion mutant (Figure 15) disappears, the mutant strain still performed well.

IreA mutation

[0125] The modification of IreA (also referred to as Ire1) is described in WO2020/112911. Shortly, the A. oryzae IreA coding sequence was modified to result in two amino acid substitutions (A80T and A83T). The modification was obtained using a 588-bp repair DNA fragment generated using primers P0200 and P0201 and genomic DNA from strain TFB-Ao0010. The repair fragment was used to co-transform strain TFB-Ao0127 using two MAD7 plasmids directing DNA cleavage at the coding region of the wt IreA sequence (using protospacers P0202 and P0203). Strain TFB-Ao0131, containing the correct sequence modification of IreA was selected.

[0126] Strain TFB-Ao0010, TFB-Ao0127 ($prtT^-$) and TFB-Ao0131 ($prtT^-$, IreA) were transformed with pTFB-0075 containing the AoBLG expression cassette that contained the Ple SP (Figure 8). Integration

of pTFB-0075 occurred by homologous recombination between the deleted niaD regions of the chromosomal locus and the 3' niaD included in the plasmid (Figure 8). Transformants containing different copies numbers were obtained. Selected transformants were used for copy number estimation. Strains obtained in the three recipient strains expressing AoBLG and having the same copy number (5) were used to compare yields. DWP cultivation for 4 days at 30° C was performed and samples from culture supernatants were run on SDS-PAGE (Figure 2).

[0127] As shown, the prtT deletion had an unexpected positive effect on AoBLG levels resulting in more than a 2-fold higher yield (strain TFB-Ao0144, Figure 2). Thus, a significant reduction of protease levels could be obtained even in strains like TFB-Ao0010/TFB-Ao0094, where genes encoding the major secreted proteases like AlpA and NprA have been inactivated, resulting in higher AoBLG yields. These results provide evidence of a prtT-controlled set of genes that code for proteases that affect the yield of recombinant protein production in already optimized fungal strains where the major proteases were inactivated.

[0128] Furthermore, the yields observed in the prtT$^-$, IreA strain TFB-Ao0145 were more than 4-fold higher compared to the non-modified strain TFB-Ao0138 (Figure 2). These results demonstrated that the IreA mutation increases the ER capacity without indication of any measurable negative effect like AoBLG mRNA degradation as described elsewhere (regulated Ire1-dependent mRNA decay (RIDD), Tanaka et al., 2023).

[0129] Overall, these results provided the basis for improved yield of recombinant BLG that were required for large scale manufacturing of this nutritionally enhanced protein to establish a sustainable process for protein production.

Example 4 – Expression of nutritionally improved BLG sequences.

[0130] As BLG is an important protein for human nutrition and protein sources are scarce, two further variant BLG proteins (SEQ ID NO: 56 and 58) encoded by synthetic genes (SEQ ID NO: 57 and 59) were expressed using the method described in Materials and Methods. These BLG proteins were enriched not only in essential amino acids (EAA) but also in branched amino acids (BCAA) compared not only to bovine BLG B (SEQ ID NO: 2) but also to the AoBLG (SEQ ID NO:4) described herein.

[0131] Using AoBLG as starting point, for each position, not having an essential amino acid, but where another BLG were found to have an essential amino acid, the non-essential amino acid was substituted with the essential amino acid. This generated the SuperBLG variant, which maintained the characteristics of a BLG, but which possessed a much higher content of EAA (107 aa or 66%) compared to AoBLG (78 or 48%) (see figure 9).

[0132] Further, also using AoBLG as starting point and using the same principle as for SuperBLG, any

position in AoBLG not having a leucine, but where another BLG were found to have a leucine, the non-leucine amino acid in AoBLG was substituted with leucine. This generated the LeuMax variant, which maintained the characteristics of a BLG, but which contained higher levels of BCAA and Leucine (Fig. 9). BLG B has 25% BCAA, while AoBLG has 26%, SuperBLG 30% and LeuMax 33%. Leucine content was maintained from BLG B to AoBLG and further to SuperBLG (22 residues or 14%) while LeuMax contains 27 Leucine residues (17%). The amount of branched amino acids in the BLGs are shown in figure 11 and alignment between AoBLG, SuperBLG and LeuMax using BLG B as reference is shown in figure 10.

Example 5. Generation of further nutritionally improved variant BLG molecules.

[0133] Identifying comprehensively modified BLG sequences which can be microbially produced is challenging (see e.g. fig. 14). One challenge to overcome was that the synthesis, folding and secretion of the BLG variant, such as SuperBLG and LeuMax could be compromised if the structure of the protein deviates extensively from BLG. Accordingly, a expression of variant BLG's having increased content of both SuperBLG and LeuMax may be significantly affected by the comprehensive number of aa changes. Using AlphaFoldstructural similarities was predicted between BLG B and AoBLG, while significant structural differences was predicted between BLG B and SuperBLG (Fig. 12). This software package was used to analyse AoBLG sequence variants containing increasing amounts of Leucine (LeuMore 1 to LeuMore 5, containing from 3 to 6 extra Leucine residues (see fig. 13). In fact, LeuMore 5 contains one more Leucine compared to LeuMax but has a much lower number of amino acid changes (6 compared to 40).

[0134] As mentioned above, multicopy strains containing either the SuperBLG or the LeuMax expression cassette produced very low amounts of the protein while significant expression of AoBLGwas detected in single copy strains (fig. 14 A). Therefore, LeuMore 1 to 5 were expressed in single copy strains, using the same strain background used for expression of AoBLG in single copy. As shown, we detected expression of all the LeuMore variants in single copy, supporting the fact that structure prediction using AlphaFold could be used for predicting protein sequences for improved expression (fig. 14 B). The expression levels varied among the LeuMore variants.

Example 6. BLG variants with increased essential amino acids content.

[0135] Additional BLG variants were designed by additional substitution of non-essential amino acid (non-EAA) residues with Leu. The design included calculation of molecular dynamics and structural stability using ΔΔG values, building structural models using AlphaFold 2 and calculating ΔΔG for all non-EAA to Leu substitutions. These substitutions were combined with amino acid changes derived from structure-based conservation. In a number of cases, the two approaches identified the same

amino acid change to Leu and resulted in a list of seven consolidated changes from either one or both approaches (S21L, D96L, E108L, Q120L, A139L, S150L and P153L). Combinations of one or more of these substitutions were used to design a new set of 50 BLG variants with increased Leu content (SEQ ID NO: 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167 and/or 169).

Example 7. BLG variants with increased essential amino acids content.

[0136] As shown in previous examples, discrete changes in the amino acid sequence of BLG can result in very different BLG protein titers (Example 5). Therefore, the expression potential for AoBLG was investigated in well-known alternative microorganisms that are industrially relevant.

Two hosts organisms were tested. One, Aspergillus niger, was a strain nowadays developed to a very advanced level which used for manufacture of numerous food proteins as described in e.g., WO 2012/160093. Another, Pichia pastoris, was a universally used, but much less optimized strain derived from the Alt-Host MIT-Industry Consortium.

[0137] For A. niger, the host strain contained both the prtT deletion and the same ireA mutation as described for A. oryzae. The same promoter, transcriptional terminator and signal peptide was used as for A. oryzae (Examples 1-5) and the same sequence encoding the AoBLG protein sequence. Multicopy strains were obtained and tested in 24 well MTP using glucose as C source (induction of expression). Thus, protein titers in A. niger are comparable to the results obtained in A. oryzae.

[0138] For P. pastoris, strain S774 was used as host. S774 is an optimized Pichia strain. It has deletions in 6 non-essential secretory protein encoding genes to enhance the production capacity for recombinant proteins. The AoBLG expression cassette used for strain construction consisted of the standard elements used in Pichia e.g., the AOX1 promoter, using the well-characterized S. cerevisiae alpha mating factor signal peptide. Transformation was performed using zeocin to facilitate multicopy strain selection. 24 well MTP cultivation was performed, using 4% glycerol for growth and 1.5 % methanol for induction as standard. SDS-PAGE of samples from culture supernatants from A. niger and P. pastoris was used for qualitative analysis of titer.

[0139] For both A. niger or P. pastoris, no optimization work for production of AoBLG (e.g., codon optimization, growth conditions, induction regime, etc.) was performed, for the preliminary screening. In a separate experiment the BLG (AoBLG) encoding genes were codon optimized for Pichia pastoris SEQ ID NO: 170) and for Aspergillus niger SEQ ID NO: 171.

[0140] As shown in figure 16, a high expression level of AoBLG in individual strains of both A. niger and P. pastoris was obtained. These results strongly indicate that 1) the use of Aspergillus sp. strains

containing the prtT and ireA modifications described here result in optimal BLG protein titers, and 2) high expression levels are also obtained in the less developed P. pastoris host strain, supporting the fact that the BLG protein sequence is universally well expressed in microbial cell factories described herein.

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Claims

1. A genetically modified microbial host cell expressing one or more heterologous genes encoding a modified beta-lactoglobulin (BLG), thereby producing the said modified BLG, wherein the modified BLG comprises at least one substitution of a non-essential and/or non-branched amino acid with an essential and/or branched amino acid compared to a parent unmodified BLG.

2. The genetically modified microbial host cell of claim 1 wherein the BLG comprises an amino acid sequence which is at least 50% identical to the BLG comprised in anyone of SEQ ID NO: 4, 163, 165, 167, 1, 2, 3, 56, 58, 60, 62, 64, 66, 68, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161 and/or 169.

3. The host cell of any preceding claim, wherein the BLG is a modified BLG, modified to have a higher fraction of essential and/or branched amino acids compared to a parent natural BLG.

4. The host cell of any preceding claim, wherein the modified BLG have an increased fraction of Leu, His and/or Val compared to a natural parent BLG.

5. The host cell of anyone of claim 3 to 4, wherein the modified BLG is a modified bovine BLG.

6. The host cell of anyone of claim 3 to 5, wherein the modified BLG comprises one or more moieties from bovine BLG A as set forth in SEQ ID NO: 1, bovine BLG B as set forth in SEQ ID NO: 2 and/or bovine BLG C as set forth in SEQ ID NO: 3.

7. The host cell of claim 6, wherein the modified BLG comprises at least one amino acid substitution compared to anyone of bovine BLG A, bovine BLG B and bovine BLG C, optionally at least two substitutions.

8. The host cell of anyone of claim 3 to 7 wherein the modified BLG comprises at least one substitution of a non-essential and/or non-branched amino acid with an essential and/or branched amino acid.

9. The host cell of claim 8 wherein the modified BLG comprises at least at least 2, such as at least 5, such as at least 10, such as at least 15, such as at least 20, such as at least 25 substitutions of a non-essential and/or non-branched amino acid with an essential and/or branched amino acid.

10. The host cell anyone of claim 8 to 9 wherein the modified BLG comprises at least at least 1, such as at least 2, such as at least 3, such as at least 4, such as 5, such as at least 6, such as at least 7 substitutions of a non-leucine amino acid with a leucine amino acid.

11. The host cell of claim 10 wherein the modified BLG comprises up to 7 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of positions 1, 5, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG as set forth in anyone of SEQ ID NO: 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167 and/or 169.

12. The host cell of claim 10 wherein the modified BLG comprises 1 substitution of a non-leucine amino acid with a leucine amino acid in a position corresponding to anyone of positions 1, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG as set forth in anyone of SEQ ID NO: 75, 77, 79, 81, 83, 85 and/or 87.

13. The host cell of claim 10 wherein the modified BLG comprises 2 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of positions 1, 5, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG as set forth in anyone of SEQ ID NO: 73, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107 and/or 109.

14. The host cell of claim 10 wherein the modified BLG comprises 3 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of positions 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG as set forth in anyone of SEQ ID NO: 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131 and/or 133.

15. The host cell of claim 10 wherein the modified BLG comprises 4 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of positions 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG as set forth in anyone of SEQ ID NO: 135, 137, 139, 141, 143, 145 and/or 147.

16. The host cell of claim 10 wherein the modified BLG comprises 5 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of positions 1, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG as set forth in anyone of SEQ ID NO: 149, 151, 153, 155, 157, 159 and/or 161.

17. The host cell of claim 10 wherein the modified BLG comprises 6 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of positions 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG as set forth in anyone of SEQ ID NO: 163, 165 and/or 167.

18. The host cell of claim 10 wherein the modified BLG comprises 7 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to position 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG as set forth in anyone of SEQ ID NO: 169.

19. The host cell of anyone of claim 7 to 10, wherein the BLG is a modified bovine BLG C comprising at least one substitution of Alanine with Valine in a position corresponding to position 118 of the bovine BLG C in SEQ ID NO: 3.

20. The host cell anyone of claim 7 to 10 wherein the BLG is a modified bovine BLG A comprising at least one substitution of Glutamine with Histidine at a position corresponding to position 59 of the bovine BLG A in SEQ ID NO: 1.

21. The host cell of claim anyone of claim 7 to 10, wherein the BLG comprises a Histidine and a Valine at positions corresponding to position 59 and 118 respectively in anyone of the BLG's comprised in SEQ ID NO: 1, 2 or 3.

22. The host cell of any preceding claim, wherein the BLG comprises an amino acid sequence as set forth in anyone of SEQ ID NO: 4, 163, 165, 167, 56, 58, 60, 62, 64, 66, 68, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161 and/or 169.

23. The host cell of any preceding claim, wherein the one or more heterologous genes encoding the BLG comprise a nucleotide sequence which is at least 50% identical to the BLG encoding gene comprised in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11, 12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126,

128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170 and/or 171.

24. The host cell of claim 23, wherein the one or more heterologous genes encoding the BLG comprise a nucleotide sequence which is the BLG encoding gene set forth in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11, 12, 57 or, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170 and/or 171.

25. The host cell of any preceding claim, wherein the one or more heterologous genes encoding the BLG further encodes a signal peptide fused to the BLG.

26. The host cell of claim 25, wherein the signal peptide is heterologous to host cell and/or to the BLG.

27. The host cell of claim 24 to 26, wherein the signal peptide comprises an amino acid sequence which is at least 50% identical to the signal peptide comprised in SEQ ID NO: 13.

28. The host cell of claim 25 to 27, comprising a nucleotide sequence encoding the signal peptide, wherein said nucleotide sequence is at least 50% identical to the signal peptide encoding sequence comprised in SEQ ID NO: 14.

29. The host cell of anyone of claim 25 to 28, wherein the signal peptide is derived from a natural defensin.

30. The host cell of claim 29, wherein the natural defensin is a fungal defensin.

31. The host cell of claim 30 wherein the fungal defensin is plectasin.

32. The host cell of claim 31, wherein the plectasin is derived from a strain of the genus of Pseudoplectania.

33. The host cell of claim 32, wherein the strain of Pseudoplectania is Pseudoplectania nigrella.

34. The host cell of any preceding claim, wherein the one or more heterologous genes encoding the BLG is under control of a promoter.

35. The host cell of claim 34, wherein the promoter is heterologous to host cell and/or to the BLG.

36. The host cell of anyone of claim 34 to 35 wherein the promoter is an engineered synthetic promoter.

37. The host cell of claim 34 to 36, wherein the promoter is an inducible promoter.

38. The host cell of claim 342 to 37 wherein the nucleotide sequence of the promoter is at least 50% identical to the promoter sequence comprised in SEQ ID NO: 15.

39. The host cell of any preceding claim, comprising one or more further genetic modifications improving the expression and/or secretion of the BLG selected from

a) attenuation, disruption and/or deletion the expression of one or more genes which are endogenous/native to the host cell; and

b) mutation of one or more genes native to the host cell.

40. The host cell of claim 39, wherein the attenuated, disrupted and/or deleted native genes encodes a protease capable of digesting BLG or its signal peptide.

41. The host cell of claim 40, wherein the protease is an alpA (alkaline protease) comprised in SEQ ID NO: 16 or 18.

42. The host cell of claim 40, wherein the protease is a nprA (neutral metalloprotease I) comprised in SEQ ID NO: 20 or 22.

43. The host cell of anyone of claim 40 to 42, wherein the native/endogenous protease is attenuated, disrupted and/or deleted by attenuation, disruption and/or deletion of a native endogenous protease transcription factor controlling the expression the one or more proteases endogenous/native to the host cell.

44. The host cell of claim 43, wherein the protease transcription factor is prtT or an analogue or ortholog thereof.

45. The host cell of claim 44, wherein the prtT is encoded by a gene having a nucleotide sequence which is at least 50% identical to the prtT sequence comprised in SEQ ID NO: 24 or 26.

46. The host cell of anyone of claim 39 to 45, wherein the mutated native genes encode an enzyme involved in activation of a transcription factor regulating the Unfolded Protein Response (UPR) in the host cell.

47. The host cell of claim 46, wherein the transcription factor is HAC1.

48. The host cell of anyone of claim 46 to 47, wherein the mutation of the enzyme increases the activation of the UPR transcription factor.

49. The host cell of anyone of claim 46 to 48, wherein the enzyme is an ER-resident transmembrane kinase and/or endoribonuclease.

50. The host cell of claim 49, wherein the enzyme is an inositol requiring enzyme.

51. The host cell of claim 50, wherein the enzyme is Ire1 or any of its analogues or orthologues.

52. The host cell of claim 51, wherein the enzyme is IreA.

53. The host cell of anyone of claim 46 to 52, wherein the enzyme has an amino acid sequence which is at least 50% identical to the inositol requiring enzyme comprised in SEQ ID NO: 28 or 30.

54. The host cell of anyone of claim 46 to 53, wherein the enzyme comprises a mutation at the positions corresponding to position A80 and/or A83 of SEQ ID NO: 28 or 30.

55. The host cell of claim 54, wherein the mutations are A80T and/or A83T.

56. The host cell of anyone of claim 50 to 53 wherein the inositol requiring enzyme is encoded by a gene having a nucleotide sequence which is at least 50% identical to the inositol requiring enzyme encoding sequence comprised in anyone of SEQ ID NO: 29 or 31.

57. The host cell of any preceding claim, comprising at least 2 copies of the one or more heterologous genes encoding the BLG.

58. The host cell of any preceding claim, wherein the one or more heterologous genes encoding the BLG are codon optimized for expression in a filamentous fungus, optionally A. Oryzae.

59. The host cell of any preceding claim further comprising one or more modifications increasing the expression, the post-translational modification, the secretion and/or the folding of the BLG.

60. The host cell of any preceding claims, wherein the microbial host cell is a eukaryotic, a prokaryotic or an algal cell.

61. The host cell of claim 60, wherein the eukaryotic cell is a fungal cell, optionally a filamentous fungus or a yeast cell.

62. The host cell of claim 61, wherein the filamentous fungus is selected from the genus of Aspergillus, Trichoderma, or Rhizopus.

63. The host cell of claim 62, wherein the filamentous fungus is selected from the species Aspergillus sp, Aspergillus oryzae, Trichoderma sp, or Rhizopus sp.

64. The host cell of claim 61, wherein the yeast is selected from the genus of Pichia, Saccharomyces, Yarrowia, or Hansenula.

65. The host cell of claim 64, wherein the yeast is selected from the species of P. pastoris, S. cerevisiae, Y. lipolytica, or Hansenula sp.

66. A genetically modified A. oryzae host cell expressing a modified BLG comprising an amino acid sequence as set forth in anyone of SEQ ID NO: 4, 163, 165, 167 56, 58, 60, 62, 64, 66, 68, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123,

125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161 and/or 169 having a higher fraction of essential amino acids compared to a parent natural BLG, said host cell expressing one or more codon optimized heterologous genes as set forth in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11, 12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170 and/or 171 encoding the modified BLG, wherein the host cell is further modified by deleting one or more native genes encoding a protease selected from the group of alpA (alkaline protease), nprA (neutral metalloprotease I), pepE (aspartic endopeptidase), and pepC (serine protease), and wherein the host cell is further modified by mutating a native inositol requiring enzyme having two mutations corresponding to positions A80T and A83T as set forth in SEQ ID NO: 28 thereby increasing activation of transcription factor HAC1 in the host cell.

67. A modified nucleotide sequence which is at least 50% identical to the BLG encoding gene comprised in anyone of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11, 12, 57, 59, 61, 63, 65, 67, 69, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 149, 150, 152, 154, 156, 158, 160, 162, 164, 167 168, 170 and/or 171 and encodes a modified BLG having at least one additional essential amino acid compared to a parent BLG.

68. A polynucleotide construct comprising the nucleotide sequence of claim 65, operably linked to an inducible promoter which is at least 50 % identical to the promoter comprised in SEQ ID NO: 15.

69. The polynucleotide construct of claim 68, wherein the construct is an expression vector.

70. The host cell of anyone of claim 1 to 66 comprising the polynucleotide construct of claims 68 to 69.

71. A cell culture, comprising the host cell of claim anyone of 1 to 65 or claim 70 and a growth medium.

72. A method for producing a BLG comprising:

a) culturing the cell culture of claim 71 at conditions allowing the cell to produce the modified BLG; and

b) optionally recovering and/or isolating the BLG.

73. The method of claims 72, further comprising one or more elements selected from:

a) culturing the cell culture in a nutrient medium;

b) culturing the cell culture under aerobic or anaerobic conditions

c) culturing the cell culture under agitation;

d) culturing the cell culture at a temperature of between 25 to 50 °C;

e) culturing the cell culture at a pH of between 3-9; and

f) culturing the cell culture for between 10 hours to 30 days.

74. The method of anyone of claim 72 to 73 wherein the recovery and/or isolation step comprises separating a liquid phase of the cell or cell culture from a solid phase of the cell or cell culture to obtain a supernatant comprising the modified BLG and/or subjecting the supernatant to one or more steps selected from:

a) separating the supernatant from the solid phase of the cell culture, such as by filtration or gravity separation;

b) contacting the supernatant with one or more adsorbent resins to obtain at least a portion of the produced BLG;

c) contacting the supernatant with one or more ion exchange or reversed-phase chromatography columns to obtain at least a portion of the BLG;

d) extracting the modified BLG; and/or

e) precipitating the modified BLG by crystallization or evaporating the solvent of the liquid phase; and optionally isolating the modified BLG by filtration or gravity separation;

thereby recovering and/or isolating the BLG.

75. A fermentation composition comprising the cell culture of claim 71 and/or the BLG comprised therein.

76. The fermentation composition of claims 75, wherein at least 50%, such as at least 75%, such as at least 95%, such as at least 99% of solid cell material has been separated from the composition.

77. The fermentation composition of anyone of claim 75 to 76, further comprising one or more compounds selected from trace metals, vitamins, salts, yeast nitrogen base, carbon source, YNB, and/or amino acids of the fermentation; wherein the concentration of the BLG is at least 1 mg/kg composition.

78. The fermentation composition of anyone of claim 75 to 77 further comprising one or more carriers, agents, additives and/or excipients.

79. A modified BLG polypeptide comprising at least one substitution of a non-essential and/or non-branched amino acid with an essential and/or branched amino acid compared to a parent unmodified BLG.

80. The modified BLG polypeptide of claim 79 comprising an amino acid sequence which is at least 50% identical to the BLG comprised in anyone of SEQ ID NO: 4, 163, 165, 167, 1, 2, 3, 56, 58, 60, 62, 64, 66, 68, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, and/or 169.

81. The modified BLG polypeptide of claim 80 comprising an amino acid sequence as set forth in SEQ ID NO: 4, 163, 165, 167, 56, 58, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, and/or 169.

82. The modified BLG polypeptide of claim 79 or 80 comprising at least at least 2, such as at least 5, such as at least 10, such as at least 15, such as at least 20, such as at least 25 substitutions of a non-essential and/or non-branched amino acid with an essential and/or branched amino acid.

83. The modified BLG polypeptide of claim 82 comprising at least 1, such as at least 2, such as at least 3, such as at least 4, such as 5, such as at least 6, such as at least 7 substitutions of a non-leucine amino acid with a leucine amino acid.

84. The modified BLG polypeptide of anyone of claim 83 comprising up to 7 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 1, 5, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone SEQ ID NO: 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167 and/or 169.

85. The modified BLG polypeptide of claim 84 comprising one substitution of a non-leucine amino acid with a leucine amino acid in a position corresponding to anyone of position 1, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone of SEQ ID NO: 75, 77, 79, 81, 83, 85 and/or 87.

86. The modified BLG polypeptide of claim 84 comprising two substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 1, 5, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone of SEQ ID NO: 73, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107 and/or 109.

87. The modified BLG polypeptide of claim 84 comprising three substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone of SEQ ID NO: 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131 and/or 133.

88. The modified BLG polypeptide of claim 84 comprising four substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone of SEQ ID NO: 135, 137, 139, 141, 143, 145 and/or 147.

89. The modified BLG polypeptide of claim 84 comprising five substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 1, 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG as set forth in anyone of SEQ ID NO: 149, 151, 153, 155, 157, 159 and/or 161.

90. The modified BLG polypeptide of claim 84 comprising six substitutions of a non-leucine amino acid with a leucine amino acid corresponding to anyone of position 21, 96, 108, 120, 139, 150 and/or 153 of the modified BLG set forth in anyone of SEQ ID NO: 163, 165 and/or 167.

91. The modified BLG polypeptide of claim 84 comprising 7 substitutions of a non-leucine amino acid with a leucine amino acid corresponding to position 21, 96, 108, 120, 139, 150 and 153 of the modified BLG set forth in SEQ ID NO: 169.

92. The modified BLG polypeptide of claim 84 comprising at least one amino acid substitution compared to anyone of bovine BLG A, bovine BLG B and bovine BLG C, optionally at least two substitutions.

93. The modified BLG polypeptide of claim 92 comprising at least one substitution of Alanine with Valine in a position corresponding to position 118 of the BLG C of SEQ ID NO: 3.

94. The modified BLG polypeptide of claim 92 comprising at least one substitution of Glutamine with Histidine at a position corresponding to position 59 of the BLG A of SEQ ID NO: 1.

95. The modified BLG polypeptide of claim 92 comprising a Histidine and a Valine at positions corresponding to position 59 and 118 respectively in anyone of the BLG's comprised in SEQ ID NO: 1, 2 or 3.

96. A food or feed composition comprising the modified bovine BLG polypeptide of anyone of claim 79 to 95.

97. A method of enriching a food or feed composition with essential amino acids comprising mixing one or more food or feed ingredients with the modified bovine BLG polypeptide of anyone of claim 79 to 95.

* * *

Figures

Figure 1

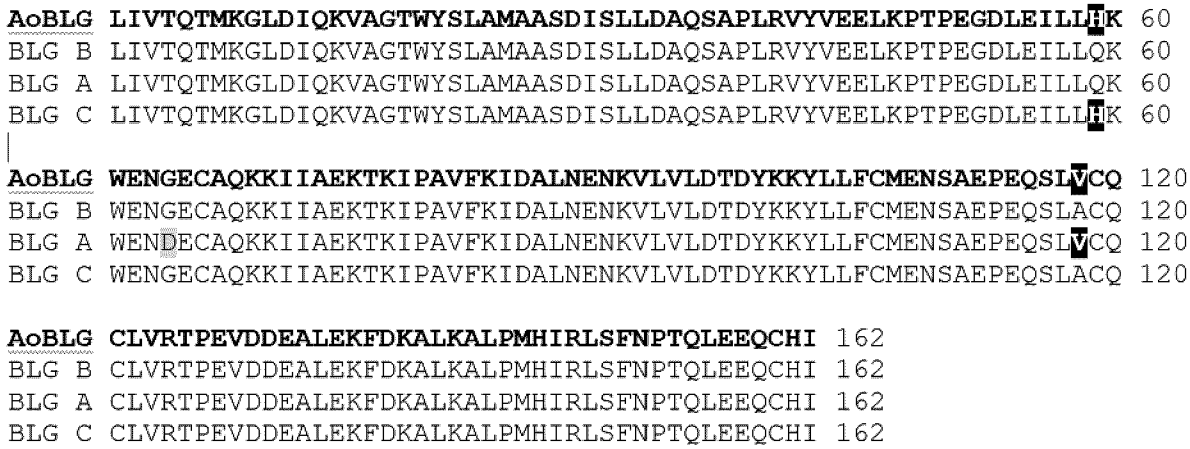


Figure 2

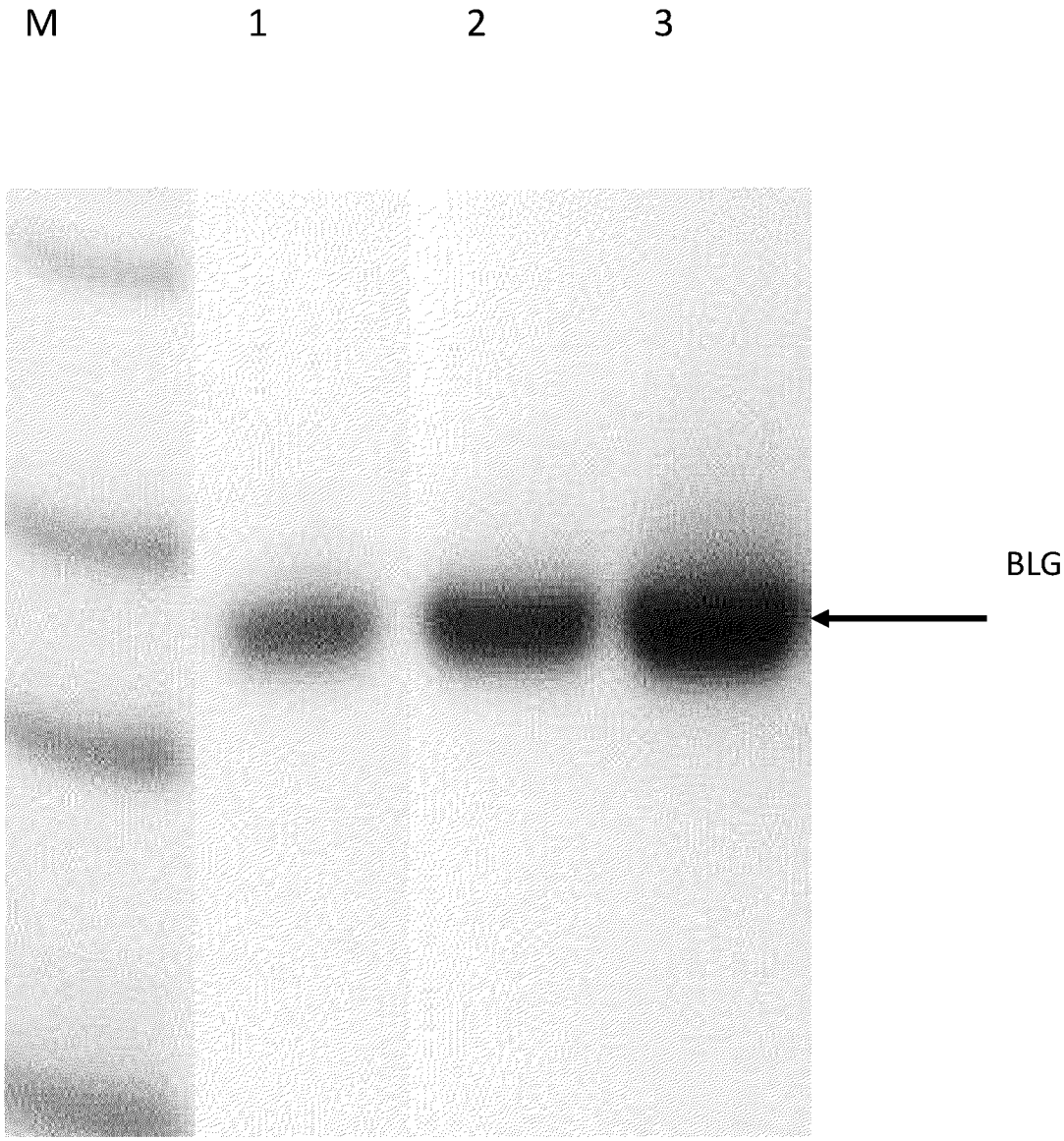
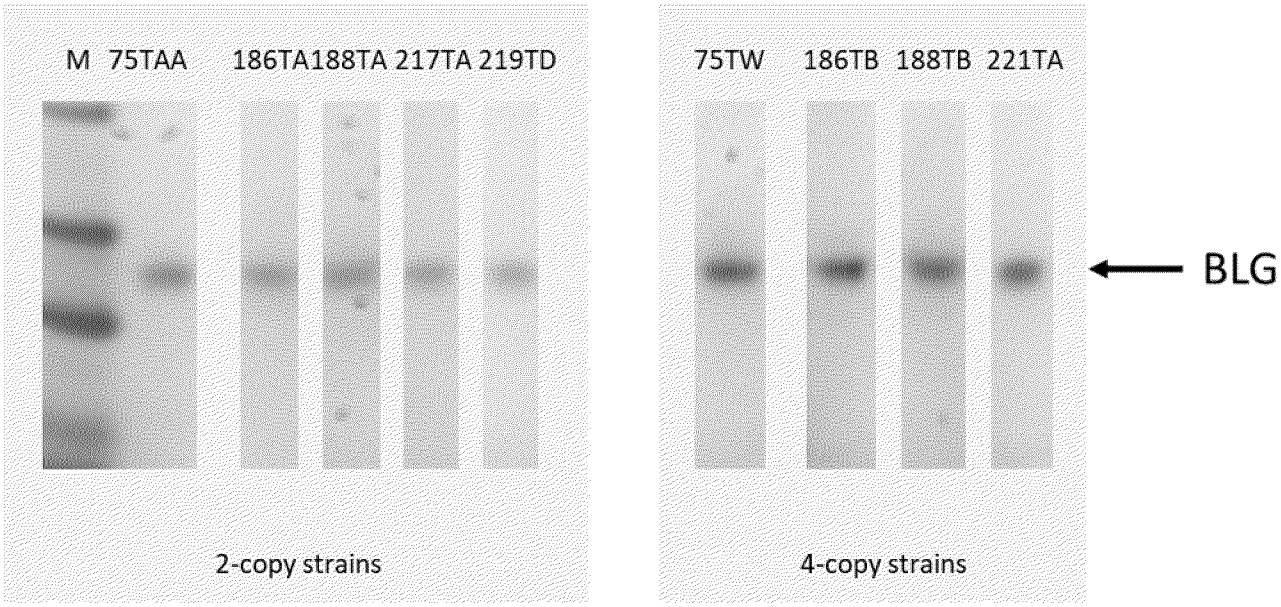


Figure 3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75TW 186TB 188TB 221TA
4-copy strains
BLG

Figure 4

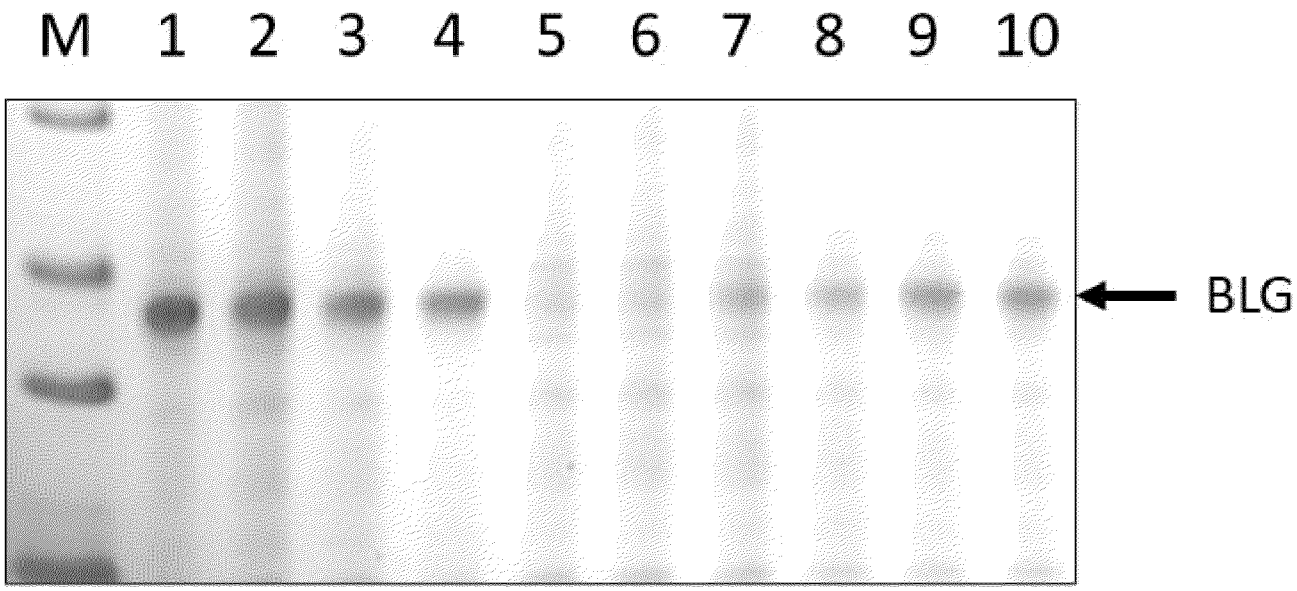
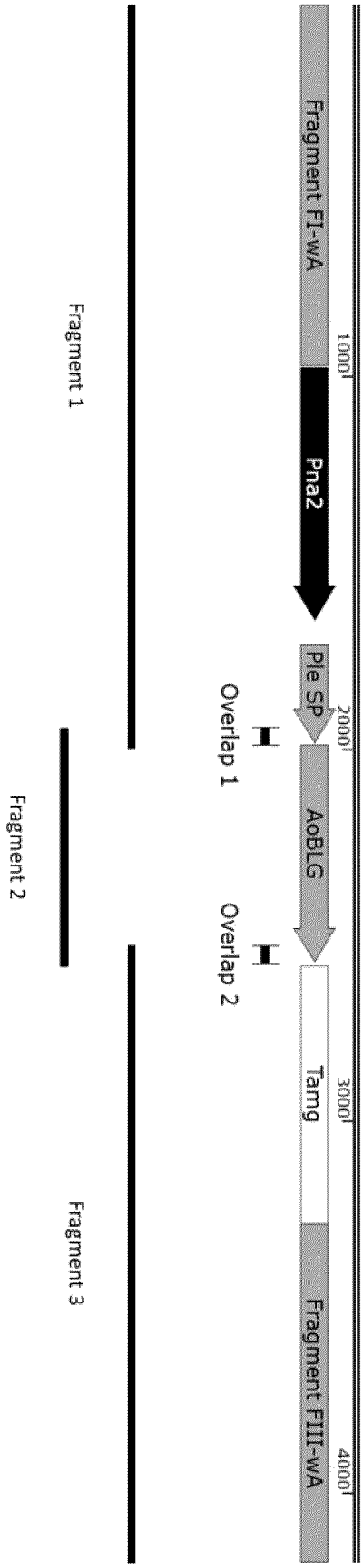


Figure 5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1000
Pna2
Ple SP
2000
AoBLG
Tamg
3000
Fragment FIII-wA
4000
Overlap 1
Overlap 2
Fragment 1
Fragment 2
Fragment 3

Figure 6

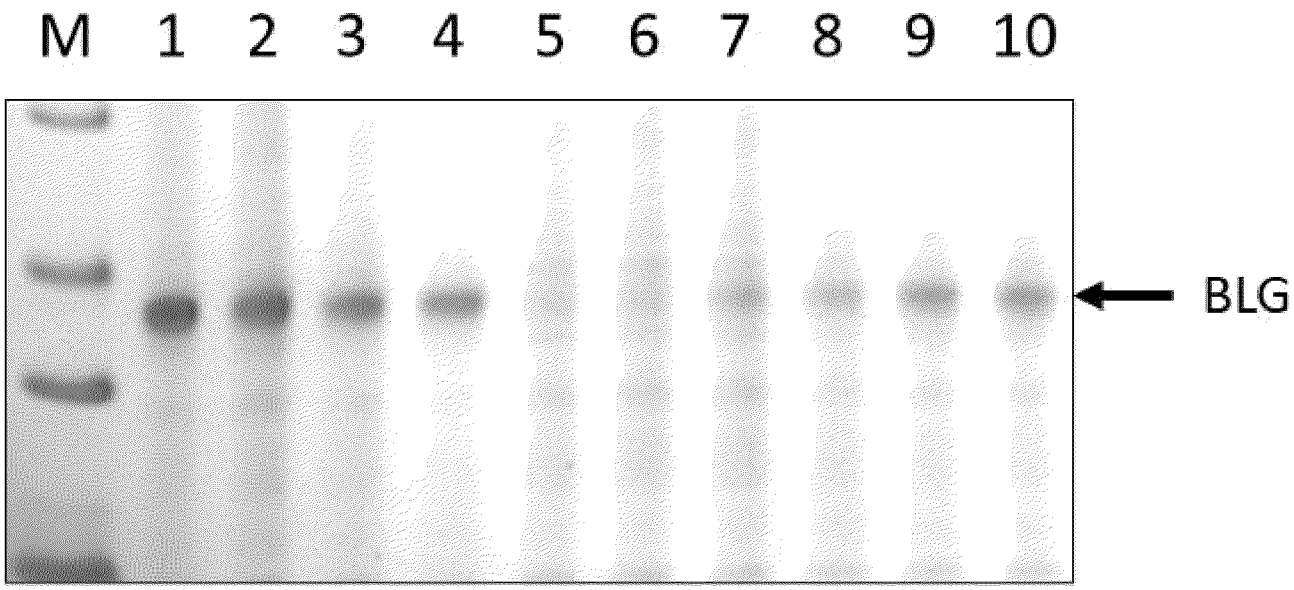
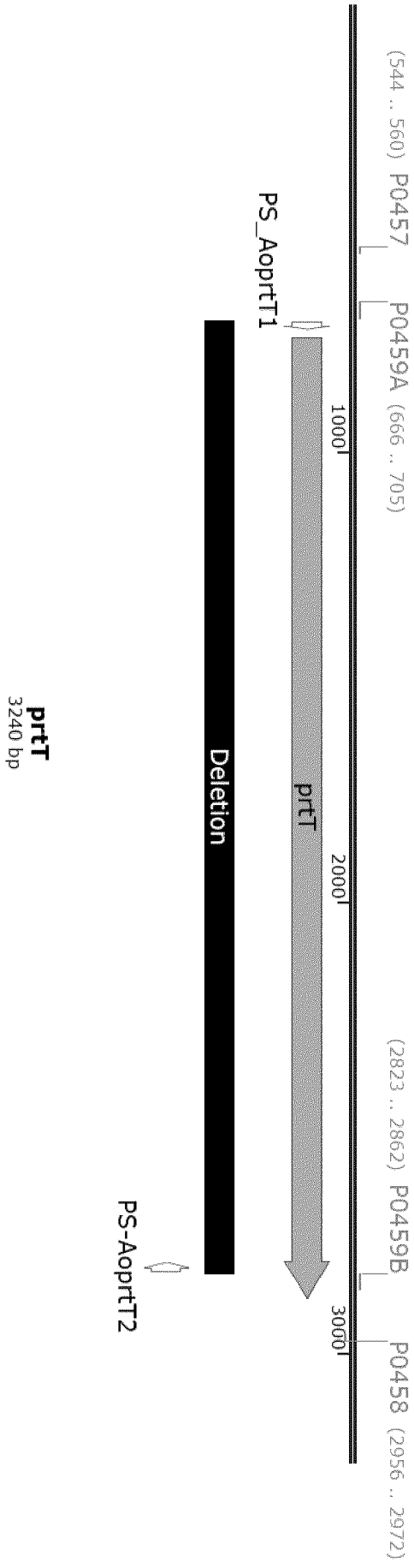


Figure 7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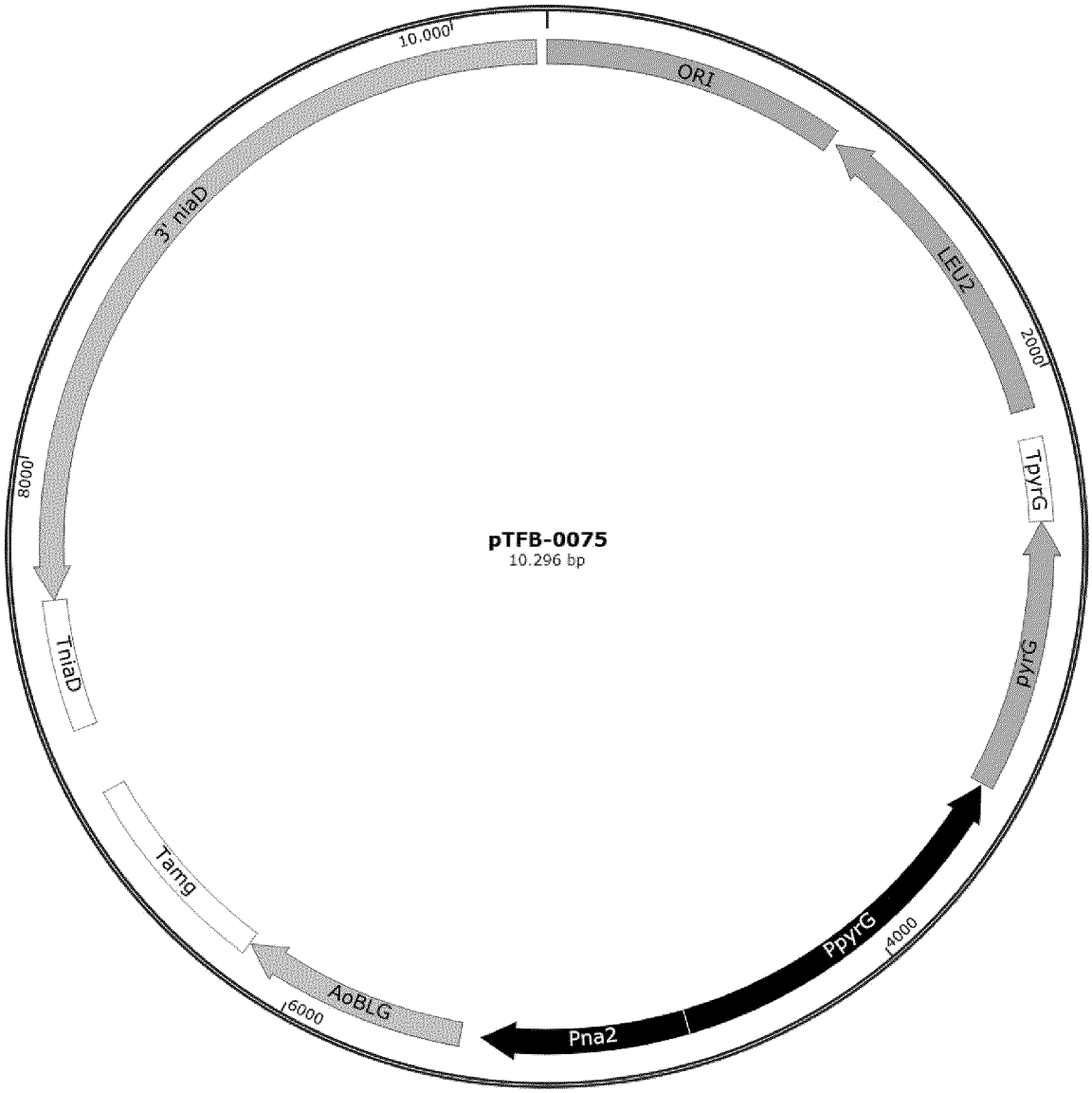
P0459A (666 .. 705)
PS_AoprtT1
1000
prtT
2000
Deletion
(2823 .. 2862) P0459B
3000
P0458 (2956 .. 2972)
PS-AoprtT2
prtT
3240 bp

Figure 8


10.000
ORI
LEU2
2000
TpyrG
pyrG
PpyrG
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AoBLG
6000
Tamg
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8000
3' niaD
pTFB-0075
10.296 bp

Figure 9

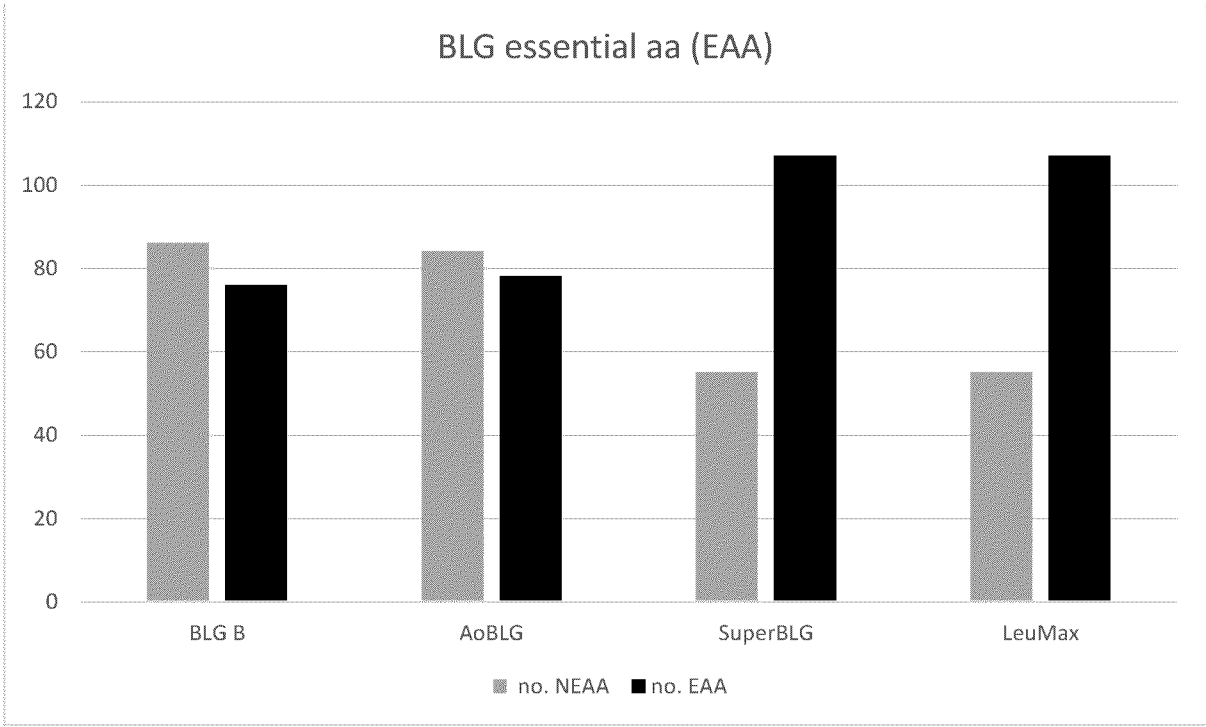
BLG essential aa (EAA)
120
100
80
60
40
20
0
BLG B
AoBLG
SuperBLG
LeuMax
no. NEAA
no. EAA

Figure 10

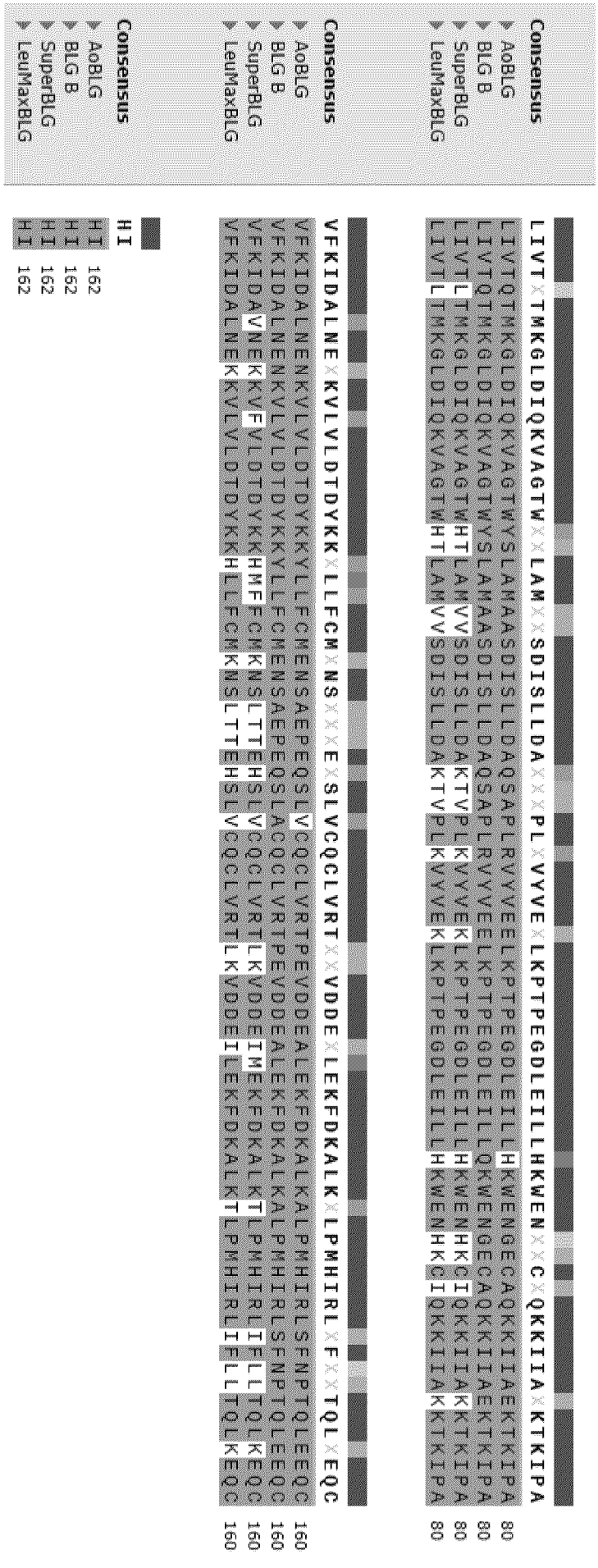
Consensus
AoBLG
BLG B
SuperBLG
LeuMaxBLG
80
Consensus
AoBLG
BLG B
SuperBLG
LeuMaxBLG
160
Consensus
AoBLG
BLG B
SuperBLG
LeuMaxBLG
162

Figure 11

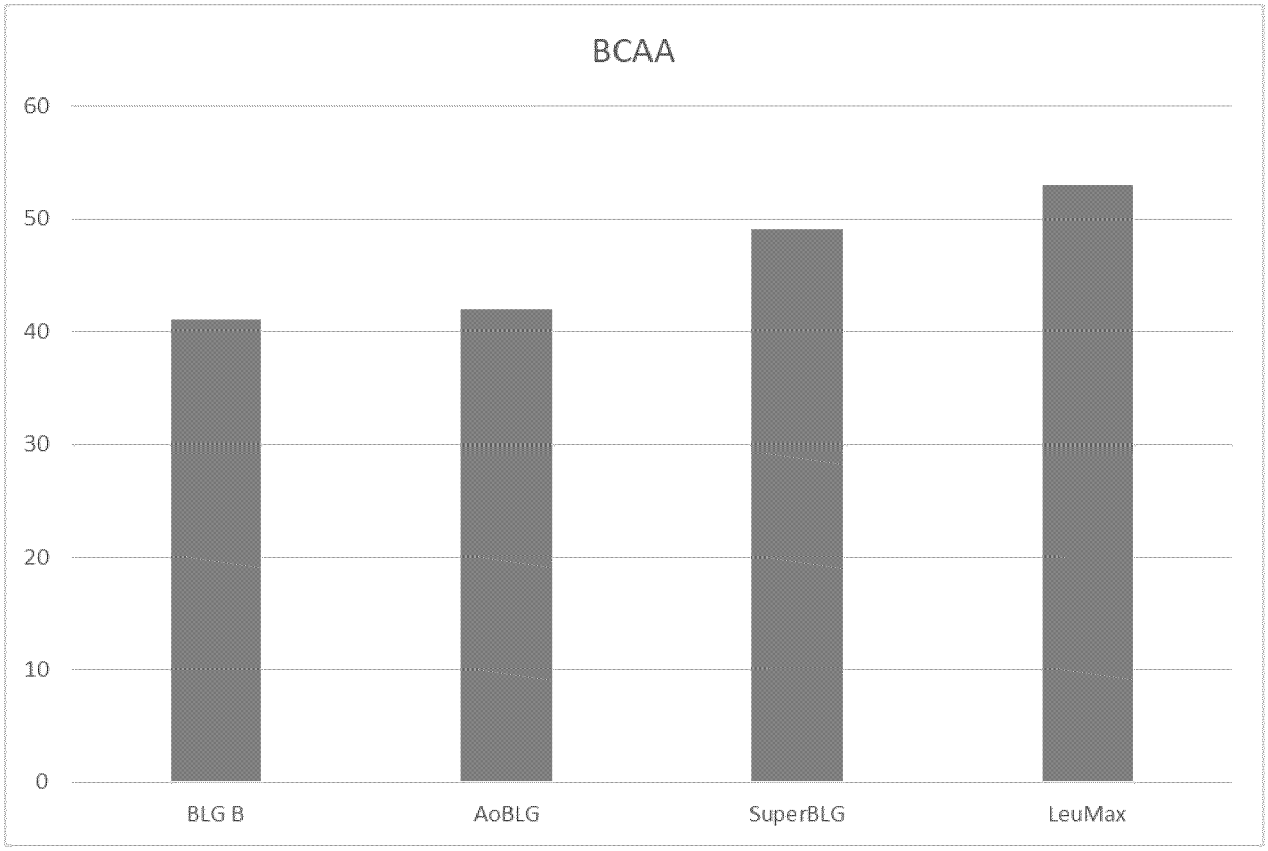

Figure 12

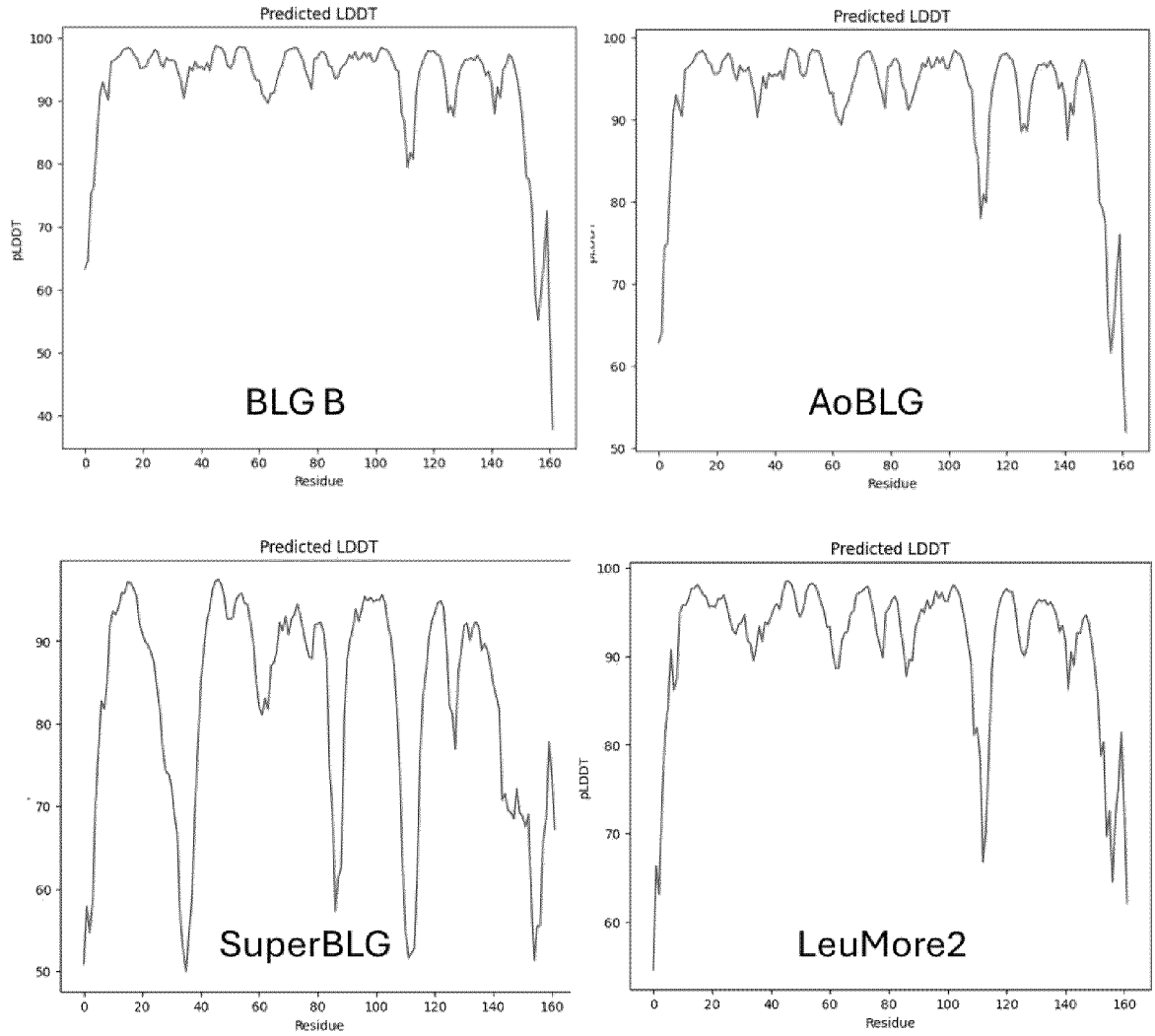
Predicted LDDT
pLDDT
Residue
BLG B
Predicted LDDT
Residue
AoBLG
Predicted LDDT
SuperBLG
Residue
Predicted LDDT
pLDDT
LeuMore2
Residue

Figure 13

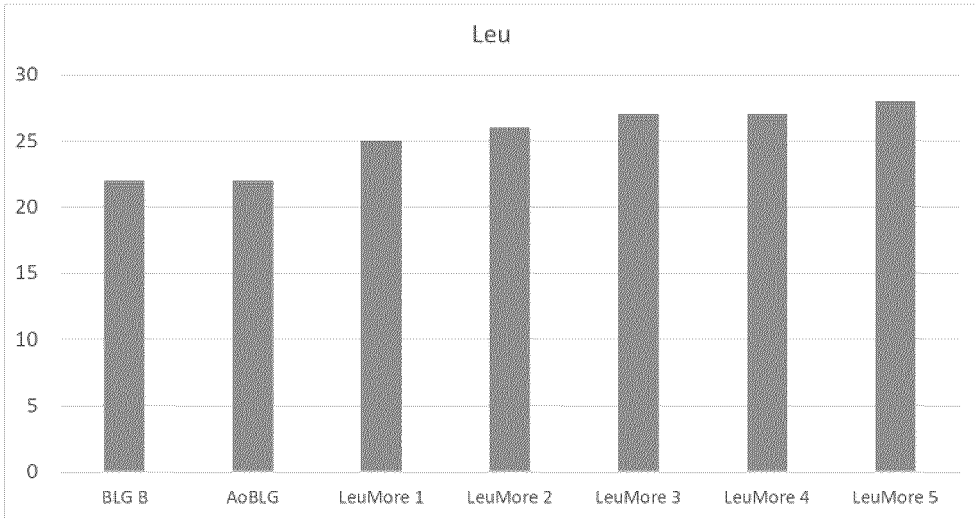
Leu
30
25
20
15
10
5
0
BLG B
AoBLG
LeuMore 1
LeuMore 2
LeuMore 3
LeuMore 4
LeuMore 5

Figure 14

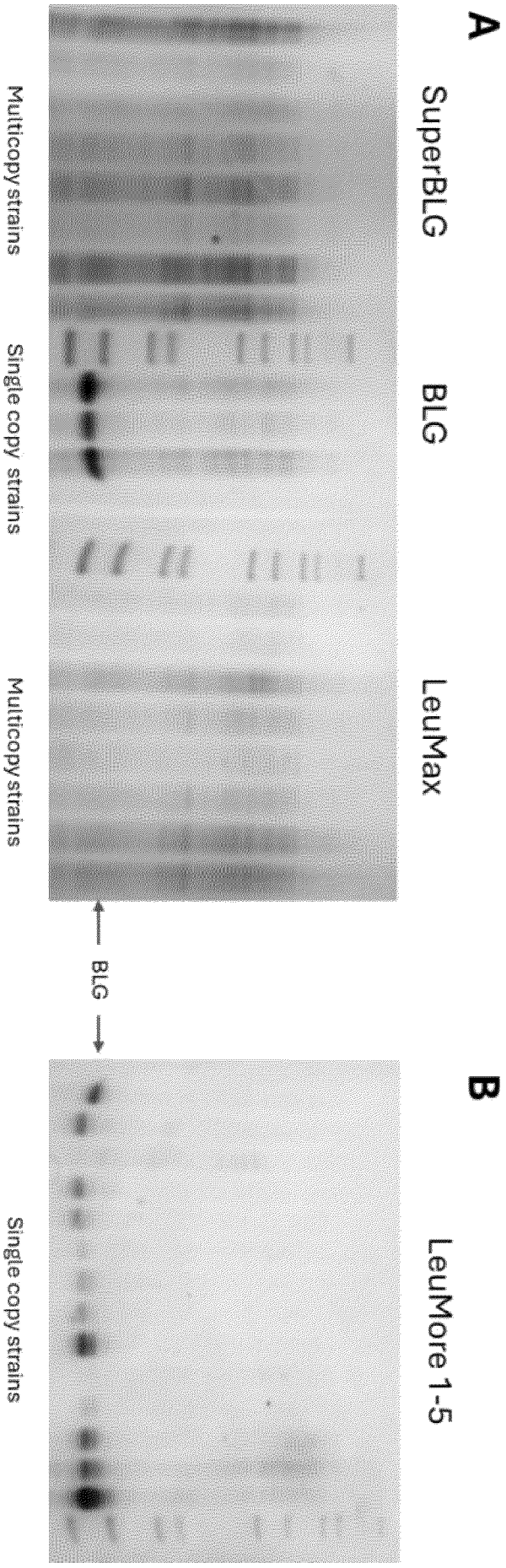
A
SuperBLG
BLG
LeuMax
Multicopy strains
Single copy strains
Multicopy strains
BLG
B
LeuMore 1-5
Single copy strains

Figure 15

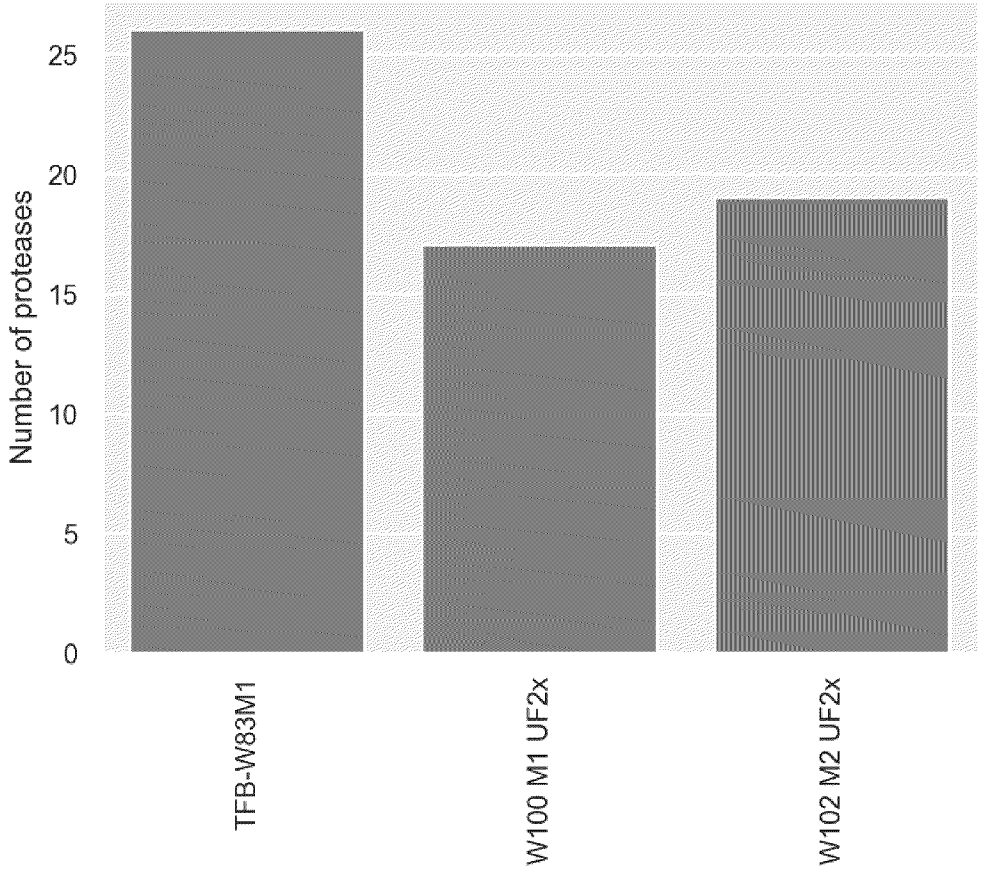
Number of proteases
25
20
15
10
5
0
TFB-W83M1
W100 M1 UF2x
W102 M2 UF2x

Figure 16