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(54) **Title:** PRODUCTION OF AN ALPHAS1-CASEIN IN A YEAST OR A FILAMENTOUS FUNGUS HOST

(57) **Abstract:** The present invention relates to a method for the extracellular production of an alphaS1- casein in a yeast or a filamentous fungus host cell and to edible composition comprising the alphaS1-casein as well as to cheese comprising predominantly alphaS1-casein.

Production of an alphaS1-casein in a yeast or a filamentous fungus host

Field of the invention

The present invention relates to the field of molecular microbiology, food technology and fermentation technology. In particular, the invention relates to a method of producing an alphaS1-casein and to edible products comprising the alphaS1-casein.

Background of the invention

In 2050 the global population will be around 10 billion people. It is generally recognized that the production of food and its ingredients needs to change significantly to keep within the agreed sustainability development goals (SDGs) for the environment and climate.

Milk, and especially cow milk, is an important source of protein and is produced all around the world (total production in 2018: 843 million tons). However, dairy production has an enormous impact on the environment. Currently over two-thirds of the world's agricultural land is used for maintaining livestock, including beef and dairy cows. Dairy cows and their manure generate significant amounts of greenhouse gas (including methane, which is a much more harmful greenhouse gas than CO_2) emissions which contribute to climate change. Water demand is very high as dairy operations consume large volumes of water to grow feed, water cows, manage manure and process products. Additionally, nitrogen emissions (from e.g. manure and fertilizer) cause worldwide major issues. Consequently, the carbon footprint and land-use factor of milk and cheese are high, even higher than that of pigs, fish and chicken. Next to these environmental and climatological aspects, also animal welfare is quite often compromised. Concerns about sustainability and animal-welfare of milk production are two important motivations for an increasing percentage of consumers to replace animal-based proteins by (vegan) plant-based protein sources such as soy, almond, pea and coconut.

Bovine milk contains around 35 g/L of caseins (i.e. 80% of the milk protein fraction) divided over alphaS1-, alphaS2-, beta- and kappa-casein within an approximate ratio of 40, 10, 40 and 10 % respectively. The four caseins are well studied in terms of amino acid composition, molecular weight, post-translational modifications (PTMs) and general physico-chemical properties. Due to the high content of prolyl residues, each casein molecule has an open and flexible conformation. Furthermore, hydrophobic and hydrophilic regions show a block distribution within the protein chain, giving each casein an amphiphilic character. Because of their nature and physico-chemical properties, caseins are unique proteins that, for many applications, cannot easily be replaced by plant-based alternatives.

Expression of recombinant mammalian caseins has previously been described in the yeasts *P. pastoris* and *S. cerevisiae* (Chung, Kun-Sub, et al. *Journal of Microbiology and Biotechnology* 1.1 (1991): 31-36; Choi, Byung-Kwon, and Rafael Jiménez-Flores. *Journal of agricultural and food chemistry* 44.1 (1996): 358-364). When producing recombinant proteins, one has to address the purification of them which is often tedious. However, this process can be greatly facilitated by the secretion of the protein in the culture broth i.e. by extracellular production.

Extracellular production has significant advantages in both analytical and industrial applications because it does not require cell disruption and has almost no contamination from host proteins. Additionally, extracellular production is particularly advantageous because the number of steps to achieve suitable purity of the products is reduced, the overall cost-effectiveness is reduced. It is thus an object of the present invention to provide for caseins that are extracellularly expressed in microbial host cells.

Summary of the invention

In one aspect, the invention provides for a method for the extracellular production of an alphaS1-casein in a yeast or a filamentous fungus host cell wherein the method comprises:

- transforming the host cell with an expression construct comprising a nucleotide sequence encoding an alphaS1-casein comprising an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 1 and comprising a deletion of the amino acids at positions 14-26 of SEQ ID NO: 1 or comprising a nucleotide sequence encoding an alphaS1-casein comprising an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 2 and comprising a deletion of the amino acids at positions 14-26 of SEQ ID NO: 2;
- culturing the cell under conditions conducive to the expression of the alphaS1-casein; and
-optionally recovery of the alphaS1-casein.

In a further aspect, the invention provides for an alphaS1-casein obtainable by the method as described herein.

In a further aspect, the invention provides for an edible composition comprising the alphaS1-casein as obtained by the method as described herein, wherein preferably the composition is a dairy product, wherein most preferably the composition is a cheese product.

In yet a further aspect, the invention provides for a cheese comprising a variant alphaS1-casein having an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 1 and comprising a deletion of the amino acids at positions 14-26 of SEQ ID NO: 1 or an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 2 and comprising a deletion of the amino acids at positions 14-26 of SEQ ID NO: 2 and at least one additional casein selected from the group consisting of naturally occurring alphaS1-casein, alphaS2-, beta- and kappa-casein or mixtures thereof, wherein relative to the total amount of caseins, the cheese comprises at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100% of the variant alphaS1-casein.

In yet a further aspect, the invention provides for a use of variant alphaS1-casein having an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 1 and comprising a deletion of the amino acids at positions 14-26 of SEQ ID NO: 1 or an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 2 and comprising a deletion of the amino acids at positions 14-26 of SEQ ID NO: 2 for the production of cheese.

Description of the invention

Definitions

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the method.

For purposes of the present invention, the following terms are defined below.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the elements is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

As used herein, the term "and/or" indicates that one or more of the stated cases may occur, alone or in combination with at least one of the stated cases, up to with all of the stated cases.

As used herein, with "At least" a particular value means that particular value or more. For example, "at least 2" is understood to be the same as "2 or more" i.e., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, ... ,etc.

The word “about” or “approximately” when used in association with a numerical value (e.g. about 10) preferably means that the value may be the given value (of 10) more or less 10% of the value.

The terms “homology”, “sequence identity” and the like are used interchangeably herein. Sequence identity is herein defined as a relationship between two or more amino acid (polypeptide or protein) sequences or two or more nucleic acid (polynucleotide) sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between amino acid or nucleic acid sequences, as the case may be, as determined by the match between strings of such sequences. "Similarity" between two amino acid sequences is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide. "Identity" and "similarity" can be readily calculated by known methods.

A "nucleic acid construct" or "nucleic acid vector" is herein understood to mean a man-made nucleic acid molecule resulting from the use of recombinant DNA technology. The term "nucleic acid construct" therefore does not include naturally occurring nucleic acid molecules although a nucleic acid construct may comprise (parts of) naturally occurring nucleic acid molecules. The terms "expression vector" or “expression construct" refer to nucleotide sequences that are capable of effecting expression of a gene in host cells or host organisms compatible with such sequences. These expression vectors typically include at least suitable transcription regulatory sequences and optionally, 3' transcription termination signals. Additional factors necessary or helpful in effecting expression may also be present, such as expression enhancer elements. The expression vector

will be introduced into a suitable host cell and be able to effect expression of the coding sequence in an in vitro cell culture of the host cell. The expression vector will be suitable for replication in the host cell or organism of the invention.

As used herein, the term "promoter" or "transcription regulatory sequence" refers to a nucleic acid fragment that functions to control the transcription of one or more coding sequences, and is located upstream with respect to the direction of transcription of the transcription initiation site of the coding sequence, and is structurally identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but not limited to transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one of skill in the art to act directly or indirectly to regulate the amount of transcription from the promoter. A "constitutive" promoter is a promoter that is active in most tissues under most physiological and developmental conditions. An "inducible" promoter is a promoter that is physiologically or developmentally regulated, e.g. by the application of a chemical inducer. An inducible promoter may also be present but not induced.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a transcription regulatory sequence is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein encoding regions, contiguous and in reading frame.

The terms "protein" or "polypeptide" are used interchangeably and refer to molecules consisting of a chain of amino acids, without reference to a specific mode of action, size, 3-dimensional structure or origin.

The term "homologous" when used to indicate the relation between a given (recombinant) nucleic acid or polypeptide molecule and a given host organism or host cell, is understood to mean that in nature the nucleic acid or polypeptide molecule is produced by a host cell or organisms of the same species, preferably of the same variety or strain. If homologous to a host cell, a nucleic acid sequence encoding a polypeptide will typically (but not necessarily) be operably linked to another (heterologous) promoter sequence and, if applicable, another (heterologous) secretory signal sequence and/or terminator sequence than in its natural environment. It is understood that the regulatory sequences, signal sequences, terminator sequences, etc. may also be homologous to the host cell. In this context, the use of only "homologous" sequence elements allows the construction of "self-cloned" genetically modified organisms (GMO's) (self-cloning is defined herein as in European Directive 98/81/EC Annex II). When used to indicate the relatedness of two nucleic acid sequences the term "homologous" means that one single-stranded nucleic acid sequence may hybridize to a complementary single-stranded nucleic acid sequence. The degree of hybridization may depend on a number of factors including the amount of identity between the sequences and the hybridization conditions such as temperature and salt concentration as discussed earlier herein.

The terms "heterologous" and "exogenous" when used with respect to a nucleic acid (DNA or RNA) or protein refers to a nucleic acid or protein that does not occur naturally as part of the

organism, cell, genome or DNA or RNA sequence in which it is present, or that is found in a cell or location or locations in the genome or DNA or RNA sequence that differ from that in which it is found in nature. Heterologous and exogenous nucleic acids or proteins are not endogenous to the cell into which it is introduced but have been obtained from another cell or synthetically or recombinantly produced. Generally, though not necessarily, such nucleic acids encode proteins, i.e. exogenous proteins, that are not normally produced by the cell in which the DNA is transcribed or expressed. Similarly exogenous RNA encodes for proteins not normally expressed in the cell in which the exogenous RNA is present. Heterologous/exogenous nucleic acids and proteins may also be referred to as foreign nucleic acids or proteins. Any nucleic acid or protein that one of skill in the art would recognize as foreign to the cell in which it is expressed is herein encompassed by the term heterologous or exogenous nucleic acid or protein.

Detailed description of the invention

The present inventors have surprisingly found that production of a specific alphaS1-casein variant in a yeast or a filamentous fungus host cell is particularly efficient and facilitates the extracellular production of this alphaS1-casein variant. The ability to produce high yields of extracellular protein is particularly advantageous as extracellular production has significant advantages in both analytical and industrial applications because it does not require cell disruption and has almost no contamination from host proteins.

Accordingly in a first aspect, the invention relates to a method for the extracellular production of an alphaS1-casein in a yeast or a filamentous fungus host cell wherein the method comprises:

- transforming the host cell with an expression construct comprising a nucleotide sequence encoding an alphaS1-casein comprising an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 1, and comprising a deletion of the amino acids at positions 14-26 of SEQ ID NO: 1 or comprising a nucleotide sequence encoding an alphaS1-casein comprising an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 2 and comprising a deletion of the amino acids at positions 14-26 of SEQ ID NO: 2; and
- culturing the cell under conditions conducive to the expression of the alphaS1-casein.

As defined herein “extracellular production” means that the host cell is capable of secreting the alphaS1-casein into the extracellular culture medium, meaning it is not necessary to disrupt the host cells to release the produced casein into the culture medium. Therefore, in the method of the invention no further chemical or temperature treatment is required to extract the alphaS1-casein from the host cell.

Therefore, in one embodiment, the method as described herein relates to a method of producing an alphaS1-casein in a yeast or a filamentous fungus host cell wherein the method comprises:

- transforming the host cell with an expression construct comprising a nucleotide sequence encoding an alphaS1-casein comprising an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 1, and comprising a deletion of the amino acids at

positions 14-26 of SEQ ID NO: 1 or comprising a nucleotide sequence encoding an alphaS1-casein comprising an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 2 and comprising a deletion of the amino acids at positions 14-26 of SEQ ID NO: 2; and

5 - culturing the cell under conditions conducive to the expression of the alphaS1-casein, wherein the alphaS1-casein is secreted into the culture medium.

"Sequence identity" and "sequence similarity" can be determined by alignment of two peptide or two nucleotide sequences using global or local alignment algorithms, depending on the length of the two sequences. Sequences of similar lengths are preferably aligned using a global alignment 10 algorithm (e.g. Needleman Wunsch) which aligns the sequences optimally over the entire length, while sequences of substantially different lengths are preferably aligned using a local alignment algorithm (e.g. Smith Waterman). Sequences may then be referred to as "substantially identical" or "essentially similar" when they (when optimally aligned by for example the programs GAP or BESTFIT using default parameters) share at least a certain minimal percentage of sequence 15 identity. GAP uses the Needleman and Wunsch global alignment algorithm to align two sequences over their entire length (full length), maximizing the number of matches and minimizing the number of gaps. A global alignment is suitably used to determine sequence identity when the two sequences have similar lengths. Generally, the GAP default parameters are used, with a gap creation penalty = 50 (polynucleotides) / 8 (proteins) and gap extension penalty = 3 (nucleotides) / 2 (proteins). For 20 nucleotides the default scoring matrix used is nwsgapdna and for proteins the default scoring matrix is Blosum62 (Henikoff & Henikoff, 1992, PNAS 89, 915-919). Sequence alignments and scores for percentage sequence identity may be determined using computer programs, such as the GCG Wisconsin Package, Version 10.3, available from Accelrys Inc., 9685 Scranton Road, San Diego, CA 92121-3752 USA, or using open source software, such as the program "needle" (using the 25 global Needleman Wunsch algorithm) or "water" (using the local Smith Waterman algorithm) in EmbossWIN version 2.10.0, using the same parameters as for GAP above, or using the default settings (both for 'needle' and for 'water' and both for protein and for DNA alignments, the default Gap opening penalty is 10.0 and the default gap extension penalty is 0.5; default scoring matrices are Blosum62 for proteins and DNAFull for DNA). When sequences have a substantially different 30 overall lengths, local alignments, such as those using the Smith Waterman algorithm, are preferred.

Alternatively, percentage similarity or identity may be determined by searching against public databases, using algorithms such as FASTA, BLAST, etc. Thus, the nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such 35 searches can be performed using the BLASTn and BLASTP programs (version 2.0) of Altschul, *et al.* (1990) J. Mol. Biol. 215:403—10. BLAST nucleotide searches can be performed with the BLASTn program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention. BLAST protein searches can be performed with the BLASTp program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to protein 40 molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST

can be utilized as described in Altschul *et al.*, (1997) Nucleic Acids Res. 25(17): 3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., BLASTp and BLASTn) can be used. See the homepage of the National Center for Biotechnology Information at www.ncbi.nlm.nih.gov/).

5 Methods of transforming yeast or a filamentous fungus host cells are known in the art. Culture conditions and conditions conducive to the expression of alphaS1-casein have been described in the art and are known to the skilled person. In certain embodiments, the medium for culturing a yeast or fungal host cell of the invention is a chemically defined medium. Typical composition of the chemically defined media for growth of (filamentous) fungi are e.g. described in 10 US 20140342396 A1, incorporated by reference herein.

In one embodiment, the method of the invention further comprises recovery of the alphaS1-casein from the fermentation broth in which the alphaS1-casein has been brought to expression. The recovery of alphaS1-casein preferably at least includes separation of the host cell biomass from the medium comprising the alphaS1-casein. One of the possibilities to separate the microbial 15 biomass is by centrifugation. Therefore, in one embodiment, the recovery is by centrifugation. However, other recovery methods are suitable, such as e.g. acid or salt precipitation and solvent extraction, as known in the art.

Preferably, at least one copy of the expression cassette is integrated in the genome of the host cell.

20 In one embodiment, host cell comprises multiple copies of the expression cassette, preferably integrated into the genome of the fungal host cell. Preferably, host cell comprises at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 25, 30 or more copies of the expression cassette, preferably integrated into the genome of the cell, more preferably at a predefined location, such as a locus comprising a highly expressed endogenous fungal or yeast gene.

25 In certain embodiments, the host cell of the invention comprises genetic modifications that reduce or eliminate the expression or activity of a vacuolar acid aspartyl protease, of at least one extracellular or cell-wall associated aspartic-type endopeptidase and/or one secreted subtilisin-type protease.

In certain embodiments, the vacuolar acid aspartyl protease, the activity or expression of 30 which is to be reduced or eliminated in the host cell, is a vacuolar acid aspartyl protease encoded by a PEP4 gene, or an orthologue thereof. In one embodiment, the PEP4 gene encodes a vacuolar acid aspartyl protease or an orthologue thereof comprising an amino acid sequence with at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 11.

In certain embodiments, the vacuolar serine-type protease, the activity or expression of which 35 is to be reduced or eliminated in the host cell is a vacuolar serine-type protease encoded by a PRB1 gene, or an orthologue thereof. In one embodiment, the PRB1 gene encodes a vacuolar serine-type protease or an orthologue thereof comprising an amino acid sequence with at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 24.

In certain embodiments, the one extracellular or cell-wall associated aspartic-type 40 endopeptidase of which the activity or expression is to be reduced or eliminated is at least one

aspartic-type endopeptidase of the yapsin family. In one embodiment, the YPS1 gene encodes an aspartic-type endopeptidase or an orthologue thereof comprising an amino acid sequence with at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 12. In one embodiment, the aspartic-type endopeptidase of the yapsin family that is reduced or eliminated is YPS'. In one embodiment, the YPS' gene encodes an aspartic-type endopeptidase or an orthologue thereof comprising an amino acid sequence with at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 26.

In one embodiment, the host cell comprises a genetic modification that reduces or eliminates the expression or activity of i) a vacuolar acid aspartyl protease encoded by a PEP4 gene as described herein, or an orthologue thereof; ii) a cell-wall associated aspartic acid protease encoded by a YPS1 gene as described herein and iii) a cell-wall associated aspartic acid protease encoded by a YPS' gene as described herein.

In certain embodiments, the secreted subtilisin-type protease, the activity or expression of which is to be reduced or eliminated in the host cell is a secreted subtilisin-type protease encoded by a SUB2 gene, or an orthologue thereof. In one embodiment, the SUB2 gene encodes a secreted subtilisin-type protease or an orthologue thereof comprising an amino acid sequence with at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 25.

In one embodiment, the host cell as described herein comprises a further genetic modification that reduces or eliminates the expression or activity of a subtilisin-like Ser-type protease. In one embodiment, the subtilisin-like Ser-type protease, the activity or expression of which is to be reduced or eliminated in the host cell is a subtilisin-like Ser-type protease encoded by a *SBT100* gene, or an orthologue thereof. In one embodiment, the *SBT100* gene encodes a subtilisin-like Ser-type protease or an orthologue thereof comprising an amino acid sequence with at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 27.

In one embodiment, the host cell as described herein comprises a further genetic modification that reduces or eliminates the level of O-linked glycosylation of casein proteins produced by the cell.

In one embodiment, the genetic modification that reduces the level of O-linked glycosylation of caseins produced by the cells is achieved by:

- eliminating or reducing the enzymatic activity of an O-mannosyl transferase; and/or
- introducing into the host cell an expression construct comprising a nucleotide sequence encoding at least one alpha-mannosidase such as alpha-1,2-mannosidase.

In one embodiment, the genetic modification comprised in the host cell that reduces or eliminates O-linked protein glycosylation, is a genetic modification that reduces or eliminates the expression or activity of at least one protein O-mannosyl transferase in the cell. The protein O-mannosyl transferase is preferably a dolichyl-phosphate-mannose-protein mannosyltransferase (EC 2.4.1.109). In one embodiment, the at least one protein O-mannosyl transferase, the activity or expression of which is to be reduced or eliminated in the host cell is an protein O-mannosyl transferase encoded by a *PMT* gene, e.g., a *PMT1* – *PMT7* gene or an orthologue thereof (see

Govindappa et al., 2013, *Protein Expression and Purification* 88(1): 164-171; Nett et al., 2013, *PLoS One* 8(7): e68325), of which *PMT1, PMT2* and *PMT4* are preferred

In one embodiment, the *PMT1* gene encodes a protein O-mannosyl transferase or an orthologue thereof comprising an amino acid sequence with at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 46. In one embodiment, the *PMT2* gene encodes an O-mannosyl transferase or an orthologue thereof comprising an amino acid sequence with at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 28. In one embodiment, the *PMT4* gene encodes an protein O-mannosyl transferase or an orthologue thereof comprising an amino acid sequence with at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO:29.

In one embodiment, reduction of O-linked glycosylation is achieved by co-expression of an alpha-mannosidase. In one embodiment the alpha-mannosidase is targeted to the secretion pathway and/or secreted by the host cell. In one embodiment the alpha-mannosidase is an alpha-1,2-mannosidase, an alpha-1,6-mannosidase, an alpha-1,3-mannosidase or a mannosidase that is able to hydrolyse multiple types of alpha-mannose linkages. More preferably, the alpha-mannosidase is an alpha-1,2-mannosidase. Accordingly, in one embodiment, the host cell as described herein further comprises an expression construct comprising a nucleotide sequence encoding at least one alpha-1,2-mannosidase. In one embodiment, the nucleotide sequence encoding the at least one alpha-1,2-mannosidase encodes at least one a protein comprising an amino acid sequence with at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 30. In other embodiments, the alpha-mannosidase may be separately produced and added to the cell culture.

In one embodiment, the level of O-linked glycosylation of caseins is reduced and/or eliminated by a genetic modification that reduces or eliminates the expression or activity of at least one O-mannosyl transferase protein in the cell such as an O-mannosyl transferase protein encoded by a PMT gene, or an orthologue thereof such as preferably PMT1, PMT2 and/or PMT4 as described herein and by additional co-expression of an alpha-mannosidase such as alpha-1,2-mannosidase in the host cell as described herein.

The host cells of the method of the invention are yeast or filamentous fungus host cells. Examples of suitable yeast host cells includes yeast from genera *Saccharomyces*, *Kluyveromyces*, *Candida*, *Komagataella, Schizosaccharomyces*, *Hansenula*, *Kloeckera*, *Schwanniomyces*, *Yarrowia*, *Kazachstania Debaryomyces* and *Naumovia*, or preferably yeast host cells of the species *Komagataella phaffii*, *Komagataella pastoris*, *Komagataella pseudopastoris Saccharomyces cerevisiae*, *Saccharomyces exiguus*, *Saccharomyces bayanus*, *Kluyveromyces lactis, Kluyveromyces marxianus, Yarrowia lipolytica* and *Schizosaccharomyces pombe*, of which *Komagataella phaffii* is most preferred. Examples of suitable filamentous fungal host cells includes fungi from genera *Alternaria, Apophysomyces, Aspergillus, Cladosphialophora, Fonsecaea, Fusarium, Lichtheimia, Mucor, Myceliophthora, Neurospora, Penicillium, Rhizopus, Rhizomucor, Trichoderma* and *Trichophyton*, or preferably filamentous fungi cells of the species *Alternaria alternata, Apophysomyces variabilis, Aspergillus* spp., *Aspergillus awamori, Aspergillus foetidus,*

Aspergillus fumigatus, Aspergillus flavus, Aspergillus oryzae, Aspergillus niger, Aspergillus nidulans, Aspergillus sojae, Aspergillus terreus, Cladosphialophora spp., *Fonsecaea pedrosoi, Fusarium* spp., *Fusarium oxysporum, Fusarium solani, Lichtheimia* spp., *Lichtheimia corymbifera, Lichtheimia ramosa, Myceliophthora* spp., *Myceliophthora thermophila, Neurospora crassa,* 5 *Penicillium chrysogenum, Penicillium simplicissimum, Penicillium brasilianum, Rhizopus* spp., *Rhizopus microsporus, Rhizomucor spp., Rhizomucor pusillus, Rhizomucor miehei, Trichoderma* spp., *Trichoderma reesei Trichophyton* spp., *Trichophyton interdigitale,* and *Trichophyton rubru*, and most preferably a species selected from *Aspergillus oryzae* and *Aspergillus niger*.

In certain embodiments, the expression construct comprises a nucleotide sequence 10 encoding an alphaS1-casein comprising the contiguous sequence of SEQ ID NO: 3 or of SEQ ID NO: 4. In certain embodiments the nucleotide sequence encodes an alphaS1-casein consisting of the amino acid sequence of SEQ ID NO: 3 or of SEQ ID NO: 4.

In certain embodiments, the expression construct comprises a nucleotide sequence encoding an alphaS1-casein having at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence 15 identity with SEQ ID NO: 1 or SEQ ID NO: 2 wherein the sequence does not comprise the stretch of amino acids EVXNENLLRFXXX (SEQ ID NO: 5), wherein X represent any naturally occurring amino acid.

In certain embodiments, the expression construct comprises a nucleotide sequence encoding an alphaS1-casein having at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence 20 identity with SEQ ID NO: 1 and does not comprise the stretch of amino acids EVLNENLLRFFVA (SEQ ID NO: 6).

In certain embodiments, the expression construct comprises a nucleotide sequence encoding an alphaS1-casein having at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with SEQ ID NO: 2 and does not comprise the stretch of amino acids EVLNENLLRFVVA 25 (SEQ ID NO: 7) or EVPNENLLRFVVA (SEQ ID NO: 8).

In certain embodiments, the expression construct comprises nucleotide sequence encoding an alphaS1-casein comprising an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 9 or 10. In certain embodiments, the expression construct comprises a nucleotide sequence encoding an alphaS1-casein comprising the contiguous 30 sequence of SEQ ID NO: 9 or of SEQ ID NO: 10. In certain embodiments the nucleotide sequence encodes an alphaS1-casein consisting of the amino acid sequence of SEQ ID NO: 9 or of SEQ ID NO: 10.

The alphaS1-casein as described in the various embodiments of the first aspect of the invention is herein also sometimes referred to as a variant alphaS1-casein, a Bovine variant A 35 casein or a Goat variant G casein.

In one embodiment, the nucleotide sequence encoding the variant alphaS1-casein as described in the various embodiments of the first aspect of the invention further encodes a secretory signal sequence. Preferably, the naturally occurring secretory signal sequence is replaced with a heterologous secretory signal sequence from a highly expressed secreted fungal protein, and

optionally a pro-sequence, operably linked in frame to the nucleotide sequence coding for the casein. In an embodiment the secretory signal sequence is heterologous to the protein of interest.

A "signal sequence" is an amino acid sequence which when operably linked to the amino-terminus of a protein of interest (i.c. a casein) permits the secretion of such protein from the host fungus. Such signal sequences may be the signal sequence normally associated with the protein of interest (i.e., a native signal sequence) or may be derived from other sources (i.e., a signal sequence foreign or heterologous to the protein of interest). Signal sequences are operably linked to a heterologous polypeptide either by utilizing a native signal sequence or by joining a DNA sequence encoding a foreign signal sequence to a DNA sequence encoding the protein of interest in the proper reading frame to permit translation of the signal sequence and protein of interest. In certain embodiments, the signal sequence is selected from the group consisting of: SEQ ID NO: 13-18.

In a second aspect, the invention relates to an alphaS1-casein as described in the various embodiments of the first aspect of the invention obtainable by the method as described herein.

In a third aspect, the invention relates to an edible composition comprising the alphaS1-casein as obtained by the method as described herein. In certain embodiments, the edible composition further comprises minerals, synthetic substances, flavoring substances (such as for examples herbs and spices), plant based proteins or proteins from microbial origin such as yeast proteins. Plant based proteins and yeast proteins suitable for the use in food products are known to the skilled person in the art. In certain embodiments, the edible composition is a food product. In certain embodiments the food product is a dairy product. A wide variety of dairy substitute products can be made using the method as described herein. Methods for producing animal-free dairy substitute products are inter alia described in WO2016/029193, which is herein incorporated by reference. Such products include without limitation, milk, whole milk, buttermilk, skim milk, infant formula, condensed milk, dried milk, evaporated milk, butter, clarified butter, cream, cottage cheese, cream cheese, creme fraiche, skyr, yogurt and various types of cheese. The dairy substitute products can also be incorporated into various food applications as a replacement for dairy products, which include ice cream, frozen custard, frozen yogurt, cookies, chocolate and cakes. In preferred embodiments, the food product is cheese.

In certain embodiments, the edible composition predominantly comprises the alphaS1-casein obtainable by the method as described. Predominant is herein defined as comprising, relative to the total amount of caseins, at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100% alphaS1-casein obtainable by the method as described. In certain embodiments, the only casein in the edible composition as described herein is the alphaS1-casein obtainable by the method as described.

In one embodiment, the invention further provides for a cheese comprising a variant alphaS1-casein as described in the various embodiments of aspect 1 of the invention and optionally at least one additional casein.

In certain embodiments, the additional casein is selected from the group consisting of naturally occurring alphaS1-casein, alphaS2-, beta- and kappa-casein or mixtures thereof. In

certain embodiments the additional caseins are of non-animal origin. In certain embodiments, the cheese as described herein, comprises relative to the total amount of caseins (wherein the total amount of caseins is defined as all alphaS1-casein (including the alphaS1-casein as described herein) alphaS2-, beta- and kappa-casein present in the cheese) at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100% of the variant alphaS1-casein as described herein.

In certain embodiments, the ratio of the variant alphaS1-casein to the at least one additional casein is from 2:1 to about 100:1.

The term "casein" is art-known and represents a family of proteins that is present in mammal-produced milk and is capable of self-assembling with other proteins in the family to form micelles and/or precipitate out of an aqueous solution at an acidic pH.

In the context of the present invention, the term “of non-animal origin” means a casein which has not been directly derived from an animal, produced from animal cells in culture, or isolated from animal products such as milk. Caseins produced by fermentation of microbes are thus “of non-animal origin” even though some products of animal origin, bacto peptone for example, can be involved during fermentation. Therefore, in the context of the invention, caseins that are naturally produced in animals will be called of non-animal origin when it is produced in a yeast or fungal cell even though its sequence or structure may be identical to the sequence or structure of the protein that would be isolated from animal. In certain embodiments, all the caseins are of non-animal origin.

Edible compositions and dairy products such as cheese, according to the invention can also contain other proteins than caseins, including whey proteins, or others. They are preferably of non-animal origin or animal-free.

In yet a further aspect, the invention provides for the use of a variant alphaS1-casein for the production of a dairy product. Methods for makings dairy products such as butter, cheese, or yogurt are well-known in the art. See, e.g., Scott, Cheesemaking Practice, Kluwer Academic/Plenum Publishers, New York, NY, 1998; U.S. Patent No. 4,360,535 (which describes methods of making creams); U.S 285,878 (which describes methods of making butter). In preferred embodiments, the invention provides for the use of a variant alphaS1-casein for the production of a cheese.

In certain embodiments, the invention provides for the use of a variant alphaS1-casein as described in the various embodiments of aspect 1 of the invention for the production of an edible composition, preferably a dairy product and more preferably a cheese that optionally comprises at least one additional casein selected from the group consisting of a naturally occurring alphaS1-casein, alphaS2-, beta- and kappa-casein or mixtures thereof. In certain embodiments the additional caseins are of non-animal origin. In certain embodiments the additional caseins are of non-animal origin. In certain embodiments, the cheese as described herein, comprises relative to the total amount of caseins, at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100% of the variant alphaS1-casein as described herein, wherein the total amount of caseins is defined as all alphaS1-casein (including the alphaS1-casein as described herein) alphaS2-, beta- and kappa-casein present in the cheese.

In certain embodiments, the ratio of the variant alphaS1-casein to the at least one additional casein is from 2:1 to about 100:1.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

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

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Description of the figures

Figure 1: Detection of extracellular bovine AlphaS1-casein variant A (fusion to the SP13 secretion signal; 24 deep-well cultivation) upon expression by a protease gene deleted (pep4- yps1-) *Pichia* strain. A) western blot analysis on several *Pichia* transformants using a rabbit polyclonal antibody against bovine AlphaS1-casein. B) analysis of same transformants via SDS-PAGE followed by Instant blue staining. Black arrow: position of secreted AlphaS1-casein.

Figure 2: Detection of bovine AlphaS1-casein variant A (fusion to different types of secretion signals; 24 deep-well cultivation) upon expression by a protease gene deleted (pep4- yps1-) *Pichia* strain. A) western blot analysis on extracellular protein. B) western blot analysis on intracellular protein. The identity of the secretion signal used is indicated on top of the blots (see Table 1 for more details). Black arrow: position of secreted AlphaS1-casein; Grey arrows: intracellular AlphaS1-casein, in some cases still attached to an unprocessed (N-glycosylated) pro-region.

Figure 3: Detection of extracellular bovine AlphaS1-casein variant A or B (fusion to the SP2 secretion signal; 1.5 L bioreactor cultivation) upon expression by a protease gene deleted (pep4- yps1-) *Pichia* strain. SDS-PAGE followed by Instant blue staining. Pp0408 and Pp0412: 5 resp. 13 copies of variant A; Pp0418 and Pp0417: 13 resp. 28 copies of variant B. Black arrow: position of secreted AlphaS1-casein.

Figure 4: Immunodetection (western blot) of extracellular bovine alphaS1-casein variant A (24 deep-well cultivation) upon expression by a protease gene deleted *Pichia* strain. A) effect of the additional knock-out of the yapsin gene YPS'; B) effect of the additional knock-out of a yapsin gene – comparison between YPS' versus YPS3, YPS" or MKC7 gene deletions. Pp0357: parental expression strain (pep4- yps1-). -P/+P: cultivation without/with addition of pepstatin A. Black arrow: position of secreted alphaS1-casein; grey arrows: alphaS1-casein degradation fragments.

Figure 5: Immunodetection (western blot) of extracellular bovine alphaS1-casein variant A (24 deep-well cultivation) upon expression by a protease gene deleted *Pichia* strain: effect of the knock-out of Ser-protease gene SBT100. Pp0487: parental expression strain (pep4- yps1- yps'-). - or +: cultivation without/with addition of PMSF. Black arrow: position of secreted alphaS1-casein; grey arrow: alphaS1-casein degradation fragment.

Figure 6: Detection of extracellular bovine alphaS1-casein variant A (24 deep-well cultivation) upon expression by a protease gene deleted *Pichia* strain. A) Western blot analysis on effect of the knock-out of PMT1 and/or alpha-1,2-mannosidase co-expression. Pp0487: parental expression strain (pep4- yps1- yps'-; 6 copies of the casein expression cassette); Pp0520: Pp0487 with PMT1 gene deletion. B) Coomassie stain analysis on effect of a PMT1 versus PMT4 gene knock-out. Parental expression strain: pep4- yps1- yps'-; >30 copies of the casein expression cassette. Black arrow: position of secreted alphaS1-casein.

Examples

Example 1 – Construction of a *Pichia pastoris* base strain by protease gene deletion

5 To reduce the chances for extracellular protein degradation, a *Pichia pastoris* (*Komagataella phaffii*) base strain was generated in which both the YPS1 (PP7435_Chr4-0387) and PEP4 (PP7435_Chr3-0072) gene were knocked out as these are known to encode a cell-wall associated and vacuolar aspartic acid protease, respectively. The gene knock-outs were generated in the commercial *Pichia pastoris* strain PPS-9010 (ATUM) using a CRISPR-mediated deletion strategy 10 and the resulting strain was designated Pp0096.

The applied CRISPR knock-out strategy was based on the Cas12a system, first described for mammalian cells and adapted in-house for use in *Pichia pastoris*. To this end, DNA fragments with the human codon-optimized Cas12a coding sequence from *Lachnospiraceae bacterium* (hLbCas12a), the human influenza hemagglutinin tag (HA), the nuclear localization signal (NLS) 15 from SV40 (NLS-2xSV40) as well as a guideRNA (gRNA) cloning site with GFP dropout cassette (pGAP-gRNA-FBP1tt with RGR-sfGFP) were generated via gene synthesis (gBlock – IDT) or oligonucleotide annealing (BioLegio). Each DNA fragment was then subcloned via BsmBI assembly into the pPTK081_0_Empty entry vector of the OPENPichia system system (distributed by GeneCorner – BCCM; Van Herpe *et al.,* 2022). Next, an 'empty' destination CRISPR/Cas12a 20 expression vector was constructed through BsaI-mediated Golden Gate Assembly of the required entry vectors belonging to or compatible with the OPENPichia toolkit. The resulting plasmid expresses the Cas12a with an in-frame N-terminal HA-tag and a C-terminal SV40 NLS under the transcriptional control of the constitutive pGPM1 promotor and AOX1 transcription terminator and further contains the empty expression cassette (GFP dropout) for the required gRNA sequences as 25 well as the hygromycin resistance marker (HygR) and an autonomous replicating sequence (ARS) for transient propagation of the plasmid within the *Pichia* cells. Then, to construct target-specific CRISPR/Cas12a vectors, the gRNA (or an array of gRNAs) for the genes of interest were designed as annealed oligonucleotides according to Zetsche *et al.* (2017) and subsequently cloned into the destination vector through BbsI-mediated Golden Gate Assembly. Due to the corresponding loss of 30 the GFP dropout cassette, a green-white screen allowed for easy identification of correctly assembled clones.

The CRISPR construct to knock-out *Pichia* PEP4 (P0249) was generated by introducing into the 'empty' CRISPR/Cas12a destination vector an array for the simultaneous expression of a first gRNA (5'-GCTTCAGCACCAATACCTAG-3' SEQ ID NO: 19) and a second gRNA sequence (5'- 35 GACCTAGGCAAAGATGCAG-3' SEQ ID NO: 20) targeting resp. a 5' and 3' sequence of the PEP4 open reading frame. The CRISPR construct to knock-out *Pichia* YPS1 (P0250) was generated by introducing into the 'empty' CRISPR/Cas12a destination vector an array for the simultaneous expression of a first gRNA (5'-AACCGTGTTATGCCCGGACT-3' SEQ ID NO: 21) and a second gRNA sequence (5'-GTAGTTATTCTCGGCGTGTG-3' SEQ ID NO: 22) targeting resp. a 5' and 3' 40 sequence of the YPS1 open reading frame. Upon transformation of the crispr plasmids, clones were selected on YPD plates containing hygromycin (200 µg/mL). Deletion of the protease genes was

confirmed via colony PCR, after which a selected deletion clone was further purified on YPD plates without selection pressure to lose the episomal Crispr plasmid containing the hygromycin resistance marker.

Example 2 – Expression of bovine AlphaS1-casein variant A by *Pichia pastoris*

A codon-optimized nucleotide sequence was ordered (gBlock – IDT) for the expression of the mature bovine AlphaS1-casein variant A in direct fusion to the SP13 signal peptide (SP13 = signal peptide from the *Pichia* protein Toh1p (gene locus PP7435_Chr2-0281); SEQ ID 23 MNLLSLTLLLFTTEALA). The sequence was flanked with the required BsmBI and BsaI sites to allow Golden Gate based modular cloning strategies using the commercially available OPENPichia plasmid system and was initially subcloned via BsmBI assembly into the pPTK081_0_Empty entry vector. The BsaI-based modular cloning strategy was used to combine the AOX1 promotor, the subcloned bovine AlphaS1-casein variant A (with direct in-frame fusion to the N-terminal SP13 signal peptide), the AOX1 transcription terminator and the zeocin resistance marker (under a weak pARG4 promotor) into a new expression cassette. The generated plasmid (P0505) was transformed to strain Pp0096. Transformants were selected based on their ability to grow on YPD (pH 8) with 100 µg/mL of zeocin and further transferred to YPD (pH 8) with 250 µg/mL of zeocin, which could induce the isolation of clones with high-copy integration of the expression cassette. Integration of the expression cassettes was confirmed via colony PCR and number of integrated copies by qPCR analysis.

PCR-positive multicopy strains were cultivated (24 deep-well format) at 28 °C for 24 hours in standard BMGY medium (pH 6.5 – 7), after which the cells were transferred into BMMY (1% methanol; pH 6.5 - 7) and cultivated for another 48 hours at 28 °C. During the methanol induction phase, pepstatin A and PMSF were added at a final concentration of 10 µg/mL resp. 2 mM). At the end of the cultivation, cells were separated from the broth and the cell-free broth was analyzed via western blot for the extracellular presence of AlphaS1-casein variant A (using a rabbit polyclonal antibody against AlphaS1-casein – custom-made at CERGroupe (Belgium) upon immunization of rabbits with in-house AlphaS1-casein purified from bovine milk). To our surprise, a significant degree of the bovine AlphaS1-casein variant A was secreted by several of the selected *Pichia* transformants (Figure 1A), while corresponding strains expressing the more common bovine AlphaS1-casein variant B did not secrete the casein despite clear intracellular accumulation (results not shown). The extracellular presence of bovine AlphaS1-casein variant A was also confirmed for the same transformants upon Instant Blue staining of an SDS-PAGE gel (Figure 1B).

Example 3 – Expression of bovine AlphaS1-casein variant A by *Pichia pastoris* using different secretion signals

The nucleotide sequence of the mature bovine AlphaS1-casein variant A was ordered synthetically (gBlock – IDT) and codon-optimized for expression by *Pichia pastoris*. The sequence was flanked with the required BsmBI and BsaI sites to allow Golden Gate based modular cloning strategies using the commercially available OPENPichia plasmid system and was initially subcloned

via BsmBI assembly into the pPTK081_0_Empty entry vector. In a next stage, the subcloned AlphaS1-casein fragment was fused in-frame to a library of 6 yeast-specific signal peptides (SEQ ID 13 to SEQ ID 18 – see Table 1). Construction of these new expression plasmids was performed via the Golden Gate based modular cloning strategy using the OPENPichia plasmid system. 3 out of the 6 tested signal peptides (ScMF, ScMF-noEAEA and Ost1) were already available within the commercial OPENPichia system, while the others were designed in-house based on data in the literature or general knowledge (Barrero *et al.*, 2018). Detailed information on the sequences and origins of the secretion signal library is presented within Table 1. All in-house designed secretion signals were ordered as double strand codon-optimized DNA fragments (gBlocks – Integrated DNA Technologies) and separately subcloned via BsmBI assembly into the pPTK081_0_Empty entry vector of the OPENPichia system.

The new plasmids (P0531-P0536 – see Table 1) combined the AOX1 promotor, a selected secretion signal, the AlphaS1-casein variant A sequence, the AOX1 transcription terminator and the zeocin resistance marker (under a weak pARG4 promotor) within individual (per secretion signal) expression cassettes. Due to the modular cloning, a 6-nucleotide sequence encoding Gly-Ser was introduced between the secretion signals and the N-terminus of the mature bovine AlphaS1-casein variant A. All 6 expression plasmids were transformed to the protease-deleted *Pichia pastoris* Pp0096 strain. Transformants were selected based on YPD (pH 8) agar plates with zeocin, as described above. Integration of the expression cassettes was confirmed via colony PCR.

Per expression construct, multiple PCR-positive strains were cultivated (24 deep-well format) at 28 °C for 24 hours in standard BMGY medium, after which the cells were transferred into BMMY (1% methanol) and cultivated for another 48 hours at 28 °C. During the methanol induction phase, pepstatin A and PMSF were added at a final concentration of 10 µg/mL resp. 2 mM). At the end of the cultivation, the broth of all selected clones was combined per expression construct. Cells were separated from the cultivation medium and both fractions were analyzed via western blot for the presence of bovine AlphaS1-casein variant A (as described above). Bovine AlphaS1-casein variant A was secreted by all the selected *Pichia* transformants (Figure 2A), but the degree of secretion was significantly lower when using the Ost1 signal peptide or the hybrid secretion signal consisting of the Ost1 signal peptide and the pro-region of the *S. cerevisiae* alpha-mating factor. Intracellular analysis nevertheless clearly indicated that there was also significant bovine AlphaS1-casein variant A expression when fused to the latter two peptide sequences (Figure 2B). Upon 24 deep-well cultivation, some of the ScMF (*S. cerevisiae* alpha-mating factor prepro-region) based expression constructs showed a similar level of bovine AlphaS1-casein variant A secretion as compared to the expression construct carrying the SP13 signal peptide.

Table 1

Plasmid nr	**SP nr**	**SP description**	**Secretion signal sequence**	**SEQ ID nr**
P0531	SP1	ScMF	MRFPSIFTAVLFAASSALAAPVNTTTE DETAQIPAEAVIGYSDLEGDFDVAVL	13

			PFSNSTNNGLLFINTTIASIAAKEEGV SLEKREAEA	
P0532	SP2	ScMF_no_EAEA	MRFPSIFTAVLFAASSALAAPVNTTTE DETAQIPAEAVIGYSDLEGDFDVAVL PFSNSTNNGLLFINTTIASIAAKEEGV SLEKR	14
P0533	SP3	ScMF_no_EAEA_Δ57-70	MRFPSIFTAVLFAASSALAAPVNTTTE DETAQIPAEAVIGYSDLEGDFDVAVL PFSASIAAKEEGVSLEKR	15
P0534	SP5	Ost1	MRQVWFSWIVGLFLCFFNVSSA	16
P0535	SP9	Ost1-ScMF pro_no_EAEA	MRQVWFSWIVGLFLCFFNVSSAAPV NTTTEDETAQIPAEAVIGYSDLEGDF DVAVLPFSNSTNNGLLFINTTIASIAAK EEGVSLEKR	17
P0536	SP25	ScMF_Δ30-43_Δ57-70_ Δ76	MRFPSIFTAVLFAASSALAAPVNTTTE DELEGDFDVAVLPFSASIAAEEGVSL EKR	18

Example 4 – Secretion of bovine AlphaS1-casein variant A versus variant B by *Pichia pastoris* upon bioreactor cultivation

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A codon-optimized nucleotide sequence was ordered (gBlock – IDT) for the expression of the mature bovine AlphaS1-casein variant A or variant B in direct fusion to the SP2 signal peptide (Table 1). Construction of the corresponding expression plasmids (plasmid P0573 for variant A; plasmid P0574 for variant B) and generation of the corresponding expression strains (using Pp0096
10 as recipient strain) was done as described above. Two strains with a different copy number for the expression cassette of the bovine AlphaS1-casein variant A (Pp0408 with 5 and Pp0412 with 13 copies) and two strains with different copy number for the expression cassette of variant B (Pp0418 with 13 and Pp0417 with 28 copies) were cultivated in 1.5 L bioreactor vessels (Dasgip).

Shake flask precultures were grown overnight at 28 °C using BMGY (1% glycerol). The total
15 preculture inoculum consisted of 5% of the bioreactor starting volume. Fermentations were performed at 24°C and the dissolved oxygen was maintained at 30%. The medium for the batch phase consisted of a minimal salts medium with 2.5% glycerol. Upon glycerol depletion, a biomass generation phase of 12 hours was initiated using a feed solution of 60% glycerol at a constant feed rate of 10.12 $mL.L^{-1}.h^{-1}$. After feed phase I, the culture was eased into methanol metabolism over a
20 six hour period while feeding 75% methanol at a rate of 0.62*t + 1.86 $mL.L^{-1}.h^{-1}$. After this six hour transition period, methanol induction continued with a constant feeding of 2.79 $mL.L^{-1}.h^{-1}$. Protease inhibitors PMSF and pepstatin A were added daily during induction. At regular time intervals, sample were taken from the bioreactors and cell-free fermentation broth was analyzed via SDS-PAGE

followed by Instant Blue staining (Figure 3). The results show that the degree of secreted Bovine AlphaS1-casein variant A significantly surpasses the amount of secreted variant B. Densitometric analysis of the casein related bands, running between 25 and 37 kDa, indicates at least a factor 4-fold difference in secreted casein levels between bovine AlphaS1-casein variant A versus B.

Example 5 - Reduction of proteolysis of bovine alphaS1-casein variant A upon secretion by Pichia pastoris

Direct gel analysis on cell-free medium samples, as well as LC-MS/MS analysis (data not shown) on bovine alphaS1-casein variant A isolated from the *Pichia* cultivation broth, indicate the occurrence of one or more proteolytic events that result in alphaS1-casein molecules with a significant N-terminal truncation. This N-terminal truncation can be partially inhibited by cultivating the *Pichia* strains in the presence of pepstatin A, which suggests that acid aspartyl protease activity is responsible for the observed degradation of alphaS1-casein. Given that the described strains are already gene-deleted for PEP4 and YPS1, the major vacuolar resp. cell-wall associated acid aspartyl proteases, additional gene knock-out studies were initiated in strain Pp0357. This strain was derived via genomic integration of plasmid P0532 into the strain Pp0096 (Δpep4, Δyps1). As a result, this strain expresses bovine alphaS1-casein variant A, fused with a Gly-Ser linker to the SP2 secretion signal, under the control of the methanol-inducible AOX1 promoter.

CRISPR-based knock-out plasmids were generated according to the methods described above to delete additional genes encoding acid aspartyl proteases, such as YPS2 (PP7435_Chr3-0919), YPS3 (PP7435_Chr3-0913), YPS7 (PP7435_Chr3-0819), MKC7 (PP7435_Chr1-0699), YPS' (PP7435_Chr3-1068) and YPS'' (PP7435_Chr3-0313; Guan *et al*. 2012, Wu *et al*. 2013). The resulting plasmids (P0420, P0423, P0421, P0425, P0471 resp. P0424) were transformed into strain Pp0357, and transformants were selected and genetically characterized as before.

For each targeted yapsin gene, at least one correct knock-out clone could be obtained within the Pp0357 strain background. These strains and the parental clone Pp0357 were cultivated (24 deep-well format) at 28°C and 200 rpm for 24 hours in standard BMGY medium pH 7, after which the cells were transferred to standard BMMY medium (1% methanol, pH 7) and cultivated for another 48 hours at 28°C and 200 rpm. During the methanol induction phase, PMSF was added at a final concentration of 2 mM. Each strain was cultivated in duplicate in either the presence or absence of 5 µg/mL pepstatin A. At the end of the cultivation, cells were separated from the broth and the cell-free broth was analyzed via western blot for the extracellular presence of (full-size) AlphaS1-casein variant A (Fig. 4A). The results show that the knock-out of the YPS' gene (SEQ ID NO: 26) significantly reduced the proteolysis of Pichia-secreted bovine AlphaS1-casein variant A as there is no longer a difference between cultivations with and without the presence of pepstatin A. Moreover, the LC-MS/MS analysis on isolated secreted AlphaS1-casein variant A confirmed the absence of a major N-terminal truncation event (data not shown). In contrast, western blot analysis clearly shows that proteolysis is still ongoing upon secretion by the parental Pp0357 strain or by the Pp0357-derived strains in which one of the other yapsin genes was deleted (Fig. 4B).

Example 6: Further reduction of proteolysis of bovine alphaS1-casein variant A upon secretion by Pichia pastoris

Despite the additional deletion of the YPS' gene, which was generated already on top of the deletion of the PEP4 and YPS1 genes, some AlphaS1-casein variant A proteolysis could still be observed (i.e. formation of a 15-20 kDa proteolytic fragment and a slight reduction in the amount of full-size protein), in particular when performing the cultivation at elevated pH (≥ 6.5) and in the absence of PMSF. These observations were indicative for Ser-type protease activity. LC-MS/MS analysis, performed on the cell-free medium of a bioreactor cultivation broth of a *Pichia* strain, which secretes bovine alphaS1-casein variant A, effectively showed the extracellular presence of Ser-type proteases (data not shown). Apart from a known vacuolar Ser-type protease, encoded by the PRB1 gene (PP7435_Chr1-0540; Wu *et al.* 2013), and a known secreted Ser-type protease, encoded by the SUB2 gene (PP7435_Chr1-1352; Salamin *et al.* 2010), a third protein, termed SBT100 (PP7435_Chr2-1137), was identified, which showed some homology to subtilisin-like Ser-type proteases.

CRISPR-based knock-out plasmid were generated according to the methods described above to delete the SBT100 gene. The resulting plasmid P0631 was separately transformed into strain Pp0487 (Δpep4, Δyps1, Δyps') and transformants were selected and genetically characterized via colony PCR. Strain Pp0487 contains up to 6 copies of the P0573 expression cassette, which consists of the AOX1 promoter, a direct fusion of the alphaMFprepro secretion signal to the N-terminus of alphaS1-casein variant A, and the AOX1TT terminator.

At least one correct knock-out clone could be selected within the Pp0487 strain background. These strains and the parental clone Pp0487 were cultivated (24 deep-well format) at 28°C and 200 rpm for 24 hours in standard BMGY medium at pH 7, after which the cells were transferred into BMMY (1% methanol; pH 7) and cultivated for another 48 hours at 28°C and 200 rpm. A clone was cultivated in duplicate in either the absence or the presence of 2 mM of PMSF during the methanol-induction phase. At the end of the cultivation, cells were separated from the broth and the cell-free broth was analyzed via SDS-PAGE for the extracellular presence of alphaS1-casein (Fig. 5). The results show that the knock-out of the SBT100 gene (SEQ ID NO: 27) significantly reduced the degradation of full-size bovine alphaS1-casein as there is no longer a clear difference between cultivations with and without the presence of PMSF. Moreover, the accumulation of the 15-20 kDa proteolytic fragment is virtually abolished, even when culturing the expression strains at pH 7 and in the absence of PMSF.

Example 7: *Reduction of O-glycosylation of bovine alphaS1-casein upon secretion by Pichia pastoris*

The extracellular analysis on cultivation broth of *Pichia* secreting bovine alphaS1-casein (variant A) shows the presence of a heterogenous casein protein population. Product-related protein bands with a higher mobility are most probably the result of proteolytic events. Apart from that, a major fraction of the extracellular AlphaS1-casein shows a reduced mobility which is most

probably related to post-translational modifications such as extended O-glycosylation (confirmed via LC-MS/MS analysis; results not shown). Hence, a CRISPR-based knock-out strategy was designed to eliminate the *Pichia* PMT1 gene (PP7435_Chr2-1095; SEQ ID NO: 29) or PMT4 gene (PP7435_Chr1-0806: SEQ ID NO: 30), coding for protein-O-mannosyltransferase involved in O-glycosylation by *Pichia pastoris* cells (Govindappa *et al.,* 2013; Nett *et al.,* 2013). The knock-out plasmids for *PMT1* or *PMT4* were transformed towards strain Pp0487 and transformants were selected and genetically characterized via colony PCR.

Another strategy to reduce O-glycosylation on *Pichia*-produced proteins is by co-expression of a secreted alpha-1,2-mannosidase as most of the *Pichia* O-glycan structures are mainly consisting of alpha-1,2-linked mannose residues (Laukens *et al.,* 2015). Using the modular OPENPichia plasmid system (Van Herpe et al., 2022), a pGAP-driven expression construct was generated for the *Trichoderma reesei* alpha-1,2-mannosidase in which the coding sequence for the mature secreted protein was fused towards the *S. cerevisiae* alpha-mating factor prepro-region as described in literature (Maras *et al.,* 1997; SEQ ID NO: 31). The resulting plasmid, P0375, contains the G418 resistance marker to allow screening of mannosidase positive transformants within zeocin-resistant alphaS1-casein expression strains. Strains Pp0487 and Pp0520, a PCR-selected PMT1 KO strain derived from Pp0487, were transformed with plasmid P0375 and clones were selected on YPD agar plates (pH 8) containing 100 µg/mL of zeocin and 500 µg/mL of G418. Integration of the mannosidase expression cassette was confirmed via colony PCR analysis.

PCR-positive clones for correct *PMT* gene knockout and/or alpha-1,2-mannosidase co-expression were cultivated in 24 deep-well format as described before, in combination with cultivations of corresponding parental strains. During the methanol induction phase, PMSF was added at a final concentration of 2 mM. At the end of the cultivation, cells were separated from the broth and the cell-free broth was analyzed via SDS-PAGE/Instant blue staining and via western blot.

The results indicate that both the PMT gene knock-out strategy as well as mannosidase co-expression can reduce the degree of O-glycosylation. Based on the gel mobility of secreted bovine alphaS1-casein variant A (Fig. 6A), the *PMT1* gene knock out (replicate cultivations of Pp0520) and the alpha-1,2-mannosidase co-expression (different individual transformants of Pp0487) result into a relatively similar reduction in protein size and heterogeneity. The latter is due to O-glycan reduction, as confirmed by mass spectrometry (RPC-UV-MS; results not shown). On top of that, the combination of both strategies appears to have a synergistic effect, resulting in a further reduction in heterogeneity and overall size of the secreted alphaS1-casein. Similar results for the PMT1 gene knock-out strategy were obtained when performed in a strain background (pep4-, yps1, yps'-) with more than 30 integrated copies of the alphaS1-casein (variant A) expression cassette (the parent strain). Finally, the knock-out of the *PMT4* gene within the parent strain also resulted in a significant reduction of the casein heterogeneity, although in a different way compared to the *PMT1* knock-out strategy (Fig. 6B). Mass spectrometry (RPC-UV-MS; results not shown) confirms that about half of the secreted alphaS1-casein contains no O-glycosylation (lowest and most intense protein band), whereas the other half still represents a heterogenous pool of O-glycosylated casein.

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Claims

1. Method for the extracellular production of an alphaS1-casein in a yeast or a filamentous fungus host cell wherein the method comprises:

5 - transforming the host cell with an expression construct comprising a nucleotide sequence encoding an alphaS1-casein comprising an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 1 and comprising a deletion of the amino acids at positions 14-26 of SEQ ID NO: 1 or comprising a nucleotide sequence encoding an alphaS1-casein comprising an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence 10 identity with SEQ ID NO: 2 and comprising a deletion of the amino acids at positions 14-26 of SEQ ID NO: 2;

- culturing the cell under conditions conducive to the expression of the alphaS1-casein; and

-optionally recovery of the alphaS1-casein.

2. The method according to claim 1, wherein multiple copies of the expression construct are 15 introduced into the host cell, preferably at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 25, 30 or more copies.

3. The method according to claim 1 or 2, wherein the yeast or a filamentous fungus host cell is selected from a genus from the group consisting of *Saccharomyces, Kluyveromyces, Candida, Komagataella, Schizosaccharomyces, Hansenula, Kloeckera, Schwanniomyces, Yarrowia,* 20 *Kazachstania, Debaryomyces, Naumovia, Alternaria, Apophysomyces, Aspergillus, Cladosphialophora, Fonsecaea, Fusarium, Lichtheimia, Mucor, Myceliophthora, Neurospora, Penicillium, Rhizopus, Rhizomucor, Trichoderma* and *Trichophyton*, wherein preferably, the host cell is selected from a species from the group consisting of *K. phaffii, K. pastoris, K. pseudopastoris S. cerevisiae, S. exiguus, S. bayanus, Kluyveromyces lactis, Kluyveromyces marxianus Y.* 25 *lipolytica, S. pombe, Alternaria alternata, Apophysomyces variabilis, Aspergillus spp.,Aspergillus awamori, Aspergillus foetidus, Aspergillus fumigatus, Aspergillus flavus, Aspergillus oryzae, Aspergillus niger, Aspergillus nidulans, Aspergillus sojae, Aspergillus terreus, Cladosphialophora spp., Fonsecaea pedrosoi, Fusarium spp., Fusarium oxysporum, Fusarium solani, Lichtheimia spp., Lichtheimia corymbifera, Lichtheimia ramosa, Myceliophthora spp., Myceliophthora thermophila,* 30 *Neurospora crassa, Penicillium chrysogenum, Penicillium simplicissimum, Penicillium brasilianum, Rhizopus spp., Rhizopus microsporus, Rhizomucor spp., Rhizomucor pusillus, Rhizomucor miehei, Trichoderma spp., Trichoderma reesei Trichophyton spp., Trichophyton interdigitale*, and *Trichophyton rubru*, of which *Komagataella phaffii* is most preferred.

4. The method according to any one of claims 1-3, wherein the expression construct comprising the 35 nucleotide sequence encoding an alphaS1-casein comprises the contiguous sequence of SEQ ID NO: 3 or of SEQ ID NO: 4.

5. An alphaS1-casein obtainable by the method as described in any one of claims 1-4.

6. An edible composition comprising the alphaS1-casein according to claim 5, wherein preferably the composition is a dairy product, wherein most preferably the composition is a cheese product.

7. The edible composition according to claim 6, wherein the only casein in the edible composition
5 is the alphaS1-casein obtainable by the method as described in any one of claims 1-4.

8. An edible composition comprising a variant alphaS1-casein having an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 1 and comprising a deletion of the amino acids at positions 14-26 of SEQ ID NO: 1 or an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 2 and comprising a
10 deletion of the amino acids at positions 14-26 of SEQ ID NO: 2 and at least one additional casein selected from the group consisting of naturally occurring alphaS1-casein, alphaS2-, beta- and kappa-casein or mixtures thereof, wherein relative to the total amount of caseins, the edible composition comprises at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100% of the variant alphaS1-casein.

15 9. Use of variant alphaS1-casein having an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 1 and comprising a deletion of the amino acids at positions 14-26 of SEQ ID NO: 1 or an amino acid sequence having at least 90, 95, 96, 97, 98, 99, or 100% sequence identity with SEQ ID NO: 2 and comprising a deletion of the amino acids at positions 14-26 of SEQ ID NO: 2 for the production of an edible composition.

20 10. Use according to claim 9, wherein the edible composition additionally comprises at least one additional casein selected from the group consisting of a naturally occurring alphaS1-casein, alphaS2-, beta- and kappa-casein or mixtures thereof and wherein relative to the total amount of caseins, the edible composition comprises at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100% of the variant alphaS1-casein.

25 11. The edible composition according to claim 8 or the use according to claim 10, wherein at least one of the at least one additional casein is of non-animal origin.

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Fig. 1A

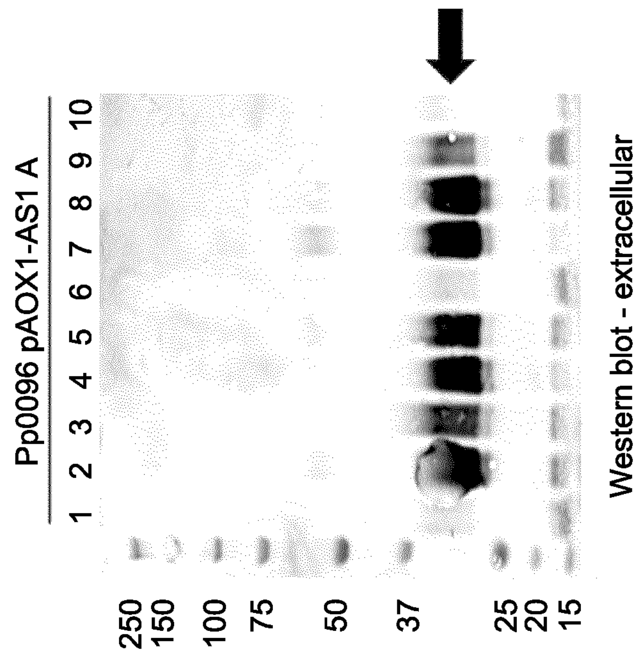
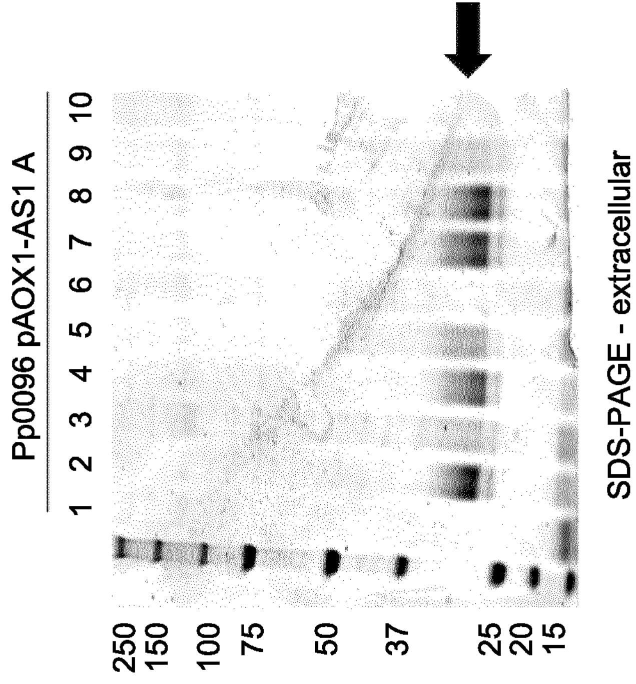


Fig. 1B



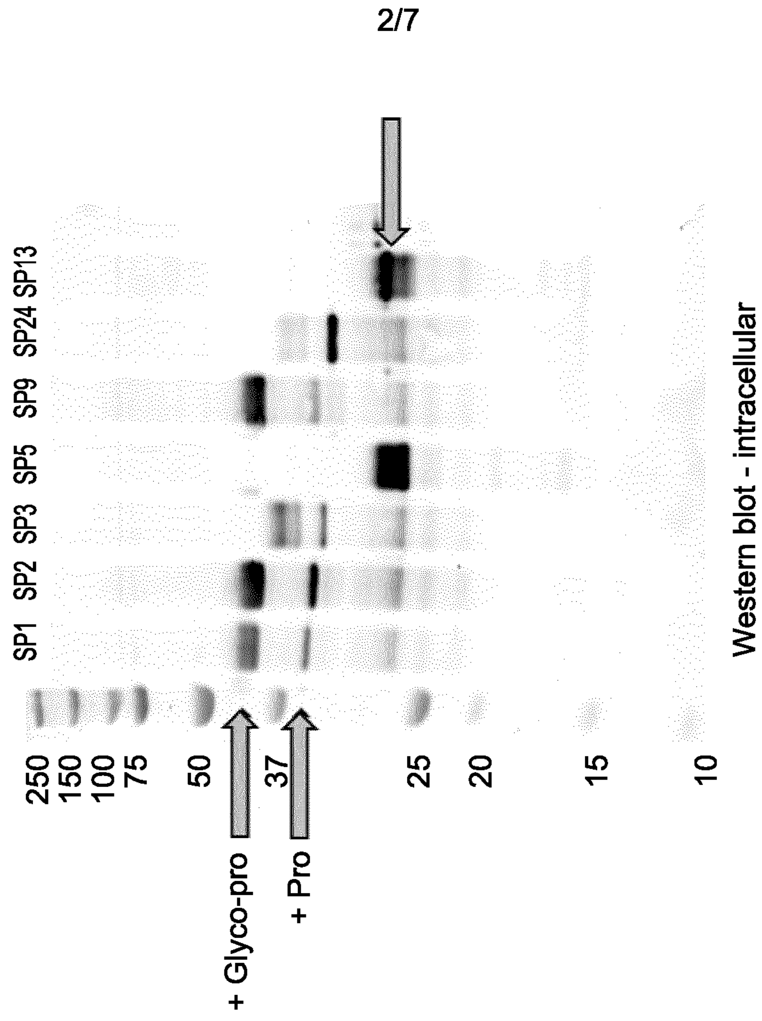


Fig. 2B

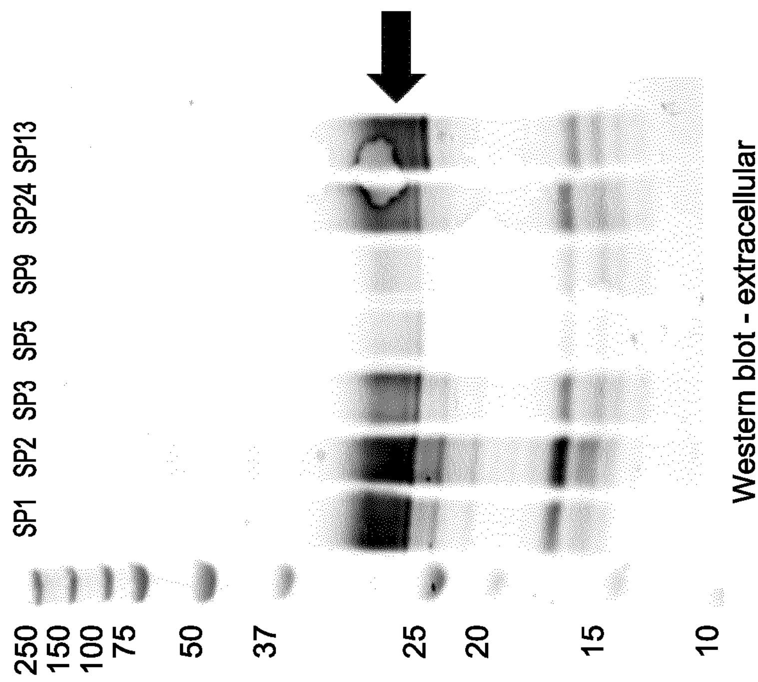
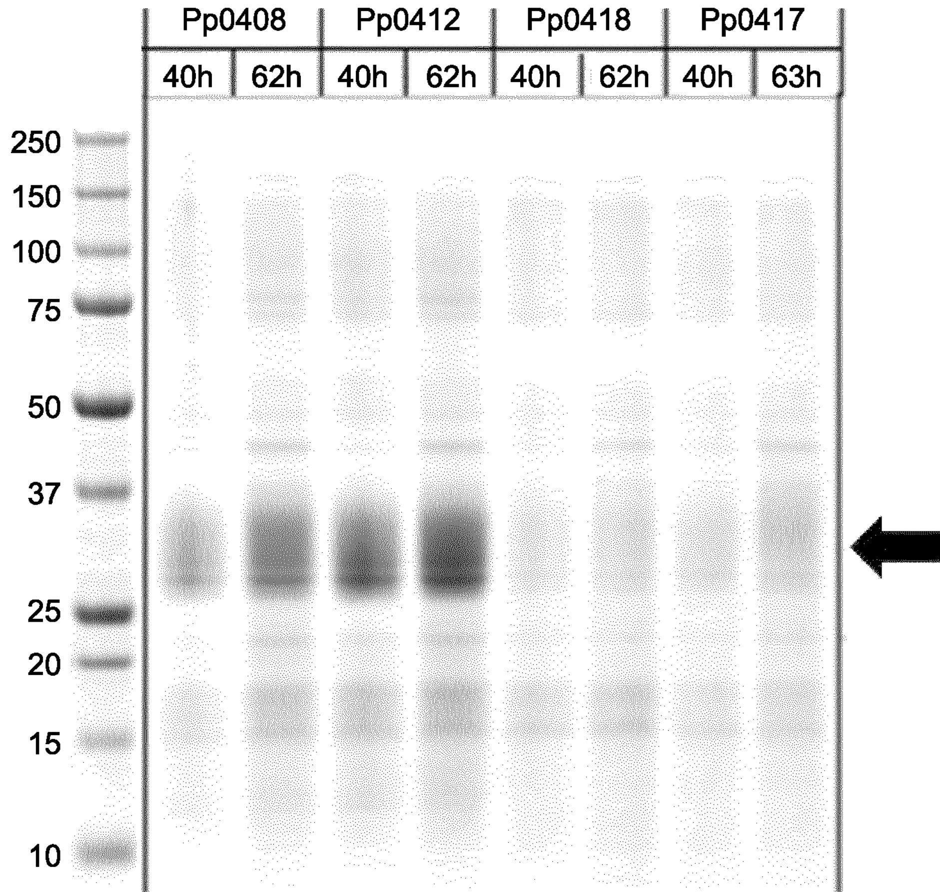


Fig. 2A

Fig. 3



SDS-PAGE - extracellular

Fig. 4A

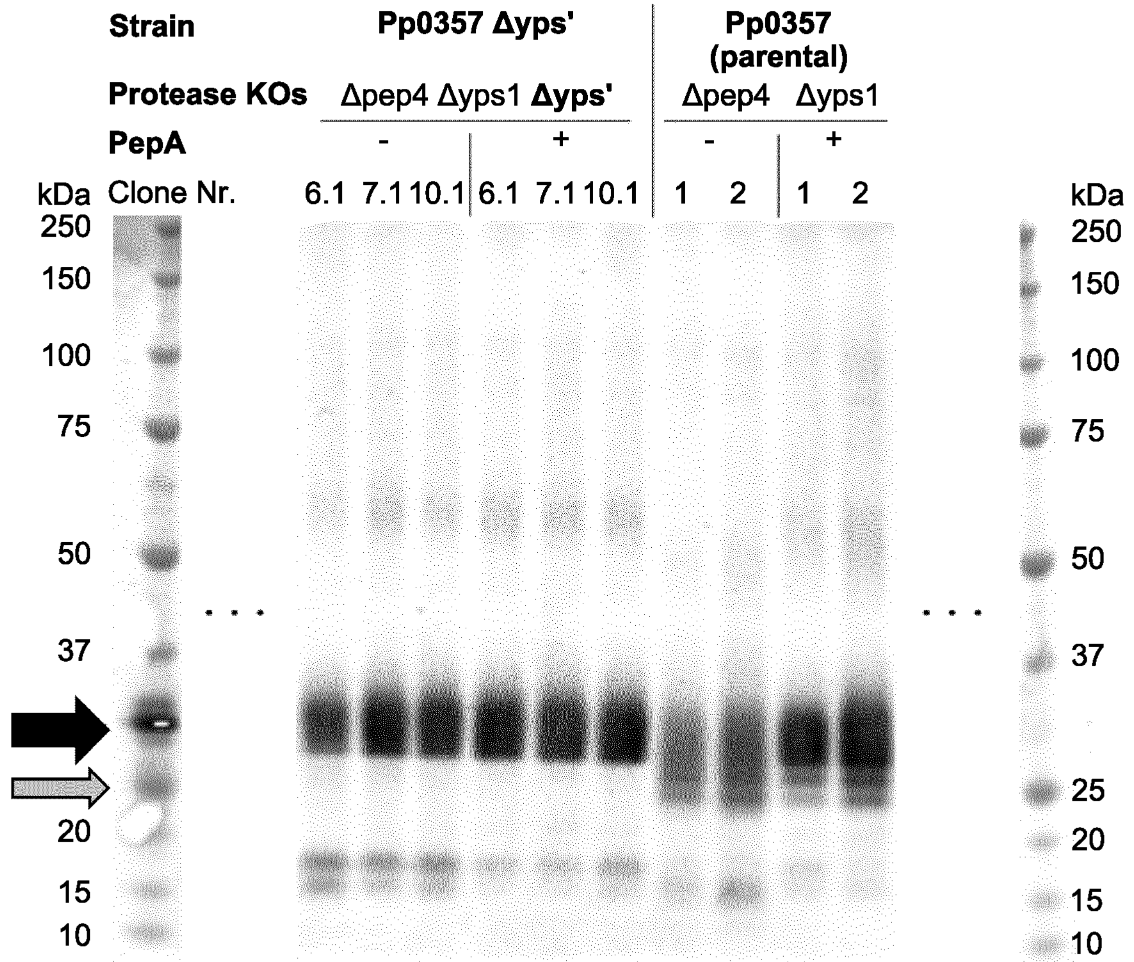


Fig. 4B

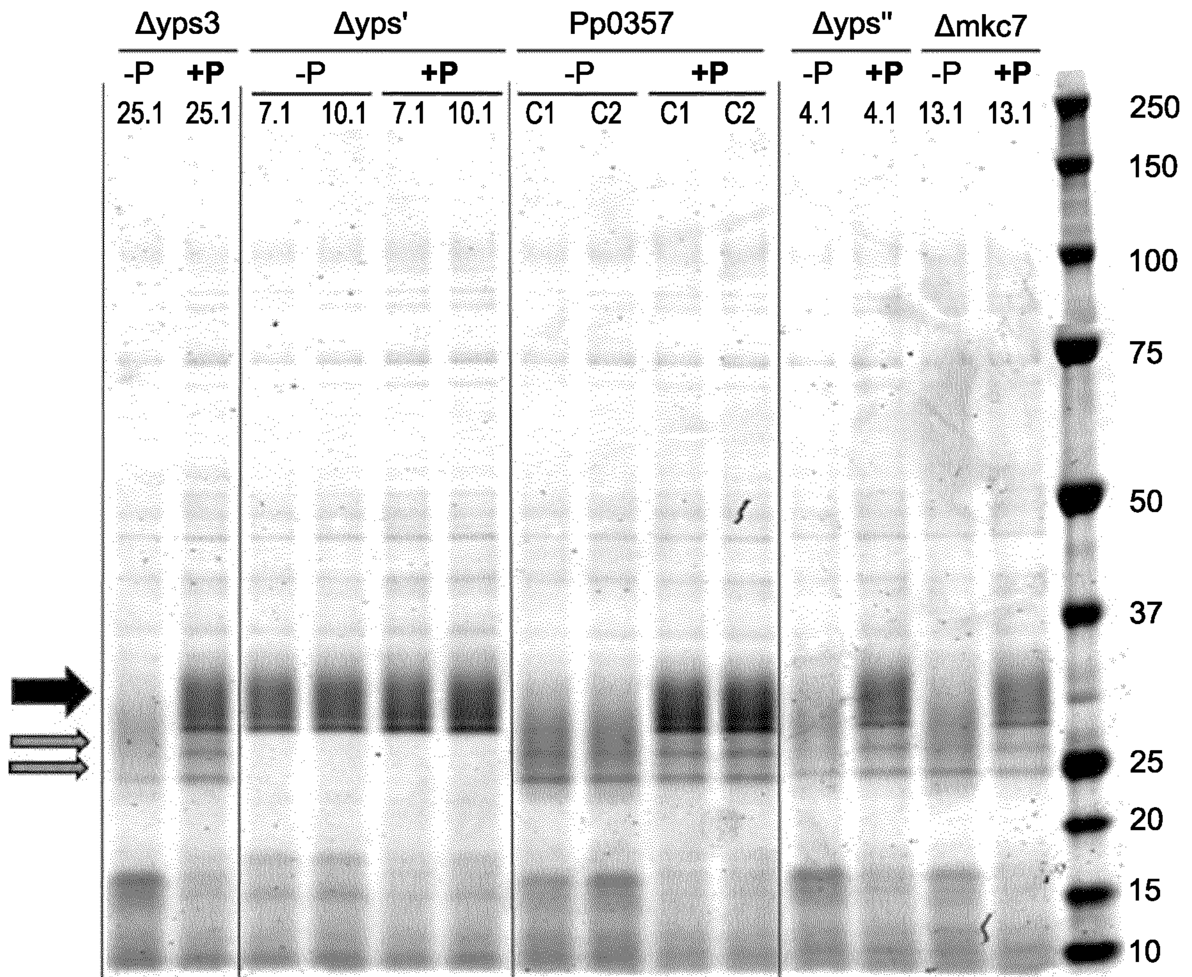


Fig. 5

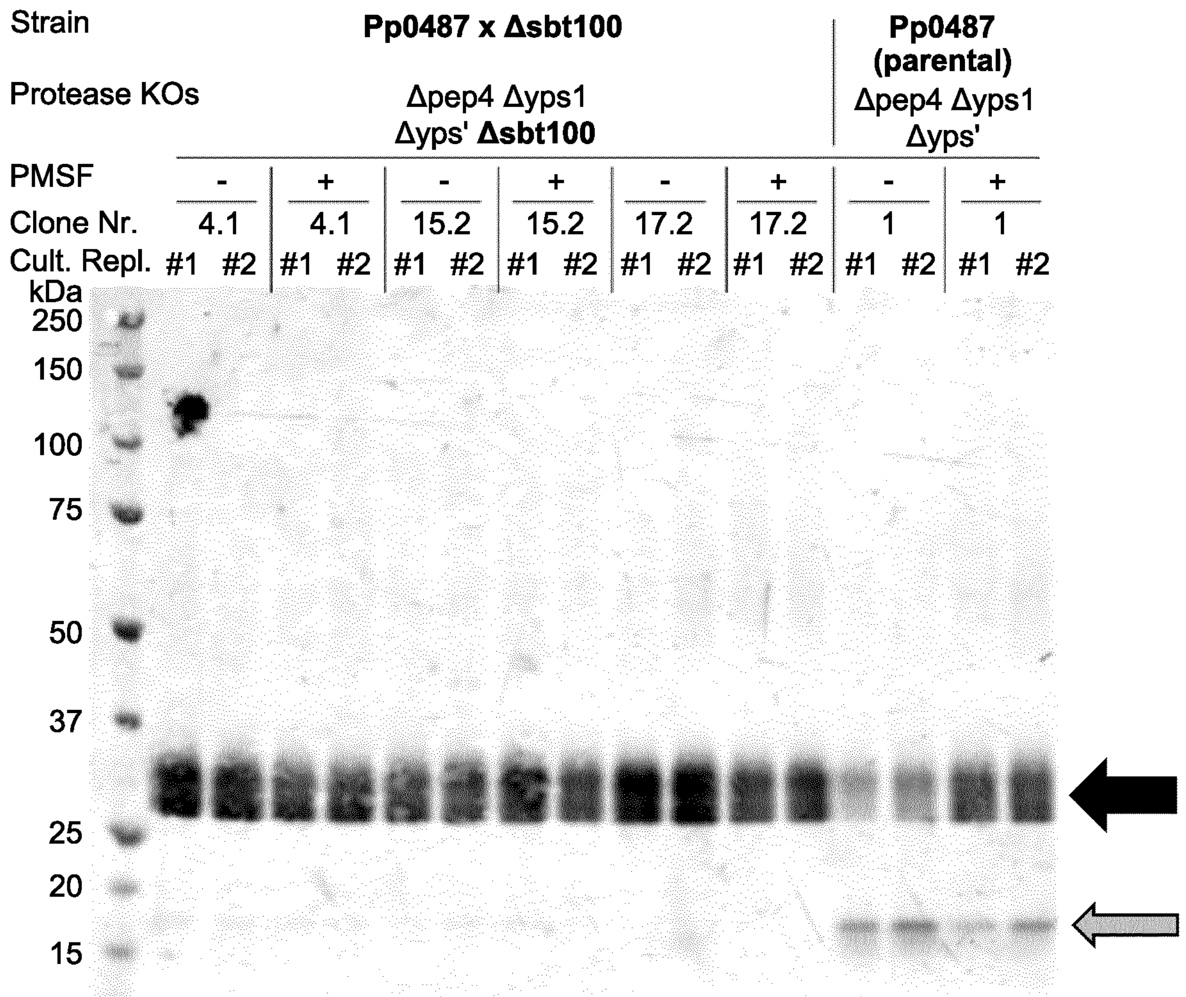


Fig. 6A

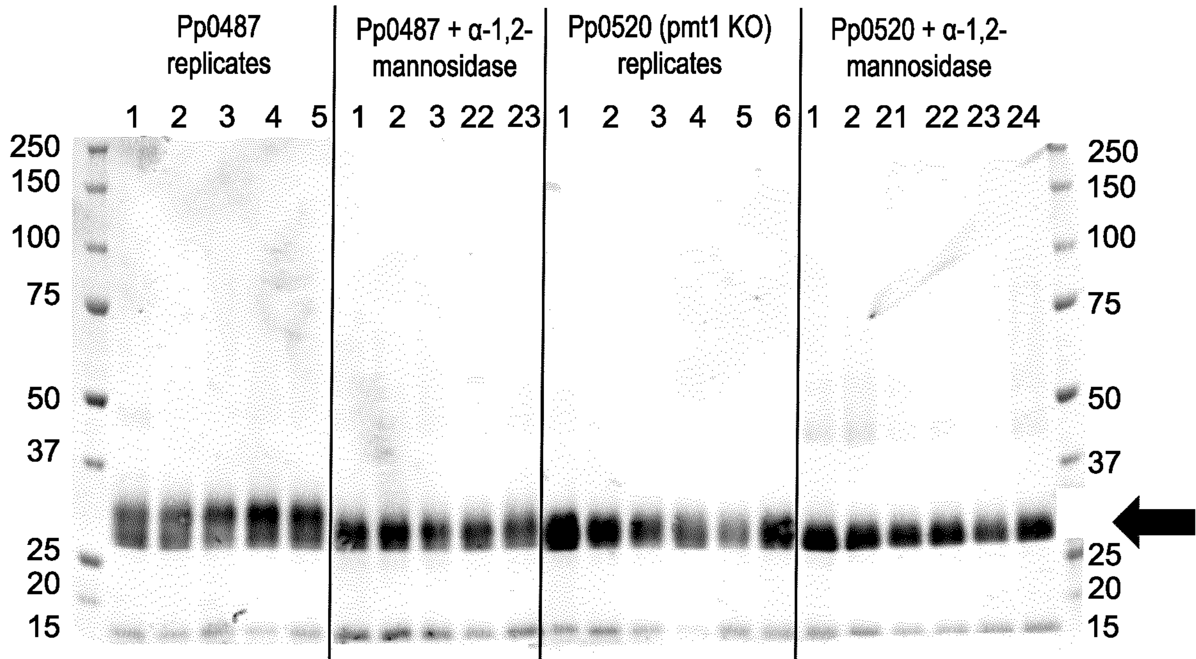
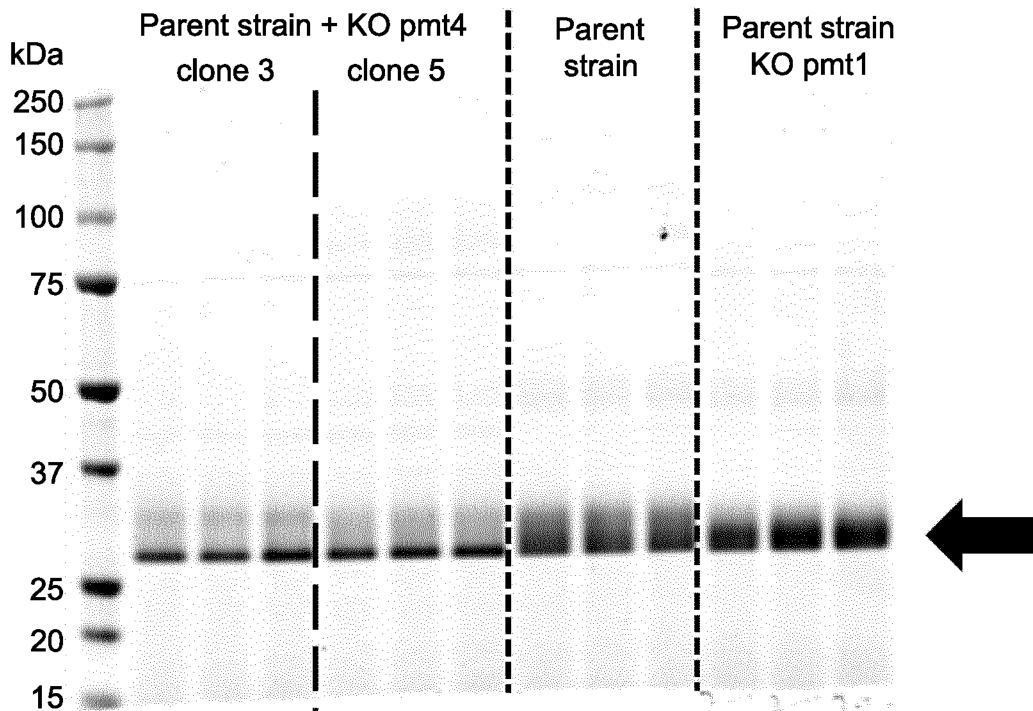


Fig. 6B



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/066507

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/47 A23C9/00 A23C11/02 A23C13/00 A23C15/00
A23C19/00 A23C19/032 A23C20/00 A23J1/00 A23J3/10
C07K14/38 C07K14/39 C12N15/63 C12N15/80 C12N15/81

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K C40B C12R A23C A23J C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, EMBASE, FSTA, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2023/074278 A1 (RADMAN INJA [US]) 9 March 2023 (2023-03-09)	1,5-11
Y	par.7-9, 11 - 12, 14, 21, 22, 113 - 117 & DATABASE Geneseq [Online] 6 April 2023 (2023-04-06), "Bos taurus alpha S1 casein fragment 24-199, SEQ 6.", retrieved from EBI accession no. GSP:BMM31713 Database accession no. BMM31713 sequence	2

X	US 2022/169690 A1 (LANQUAR VIVIANE [US] ET AL) 2 June 2022 (2022-06-02)	1,3-7
Y	par.8, 334, 366, 367, 402, 1093	2,8-11
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[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 September 2024	01/10/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bonello, Steve

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	& DATABASE Geneseq [Online] 14 July 2022 (2022-07-14), "Bos taurus alpha S1 casein, SEQ 355.", retrieved from EBI accession no. GSP:BLE16650 Database accession no. BLE16650 sequence -----	
Y	US 2023/141532 A1 (GIBSON MATT [US] ET AL) 11 May 2023 (2023-05-11) par.6,83,86,186,191,133,196 -----	1-11
Y	WO 2022/038601 A1 (RE MILK LTD [IL]) 24 February 2022 (2022-02-24) p.2 l.21, p.11 l.21, p.13 l.12, p.30 l.7-20 -----	1-11

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Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2024/066507

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