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(54) Title: ENDOPROTEASE COMPOSITION

(57) Abstract: A composition comprising or consisting of: - an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and - a solution stabilizer and/or an osmolyte; and - optionally a solvent.

ENDOPROTEASE COMPOSITION

Field of the invention

5 [001] The invention relates to a novel endoprotease composition useful for transporting endoprotease enzyme.

Background of the invention

[002] Haze is a well known phenomenon in the beverage industry. Haze can for example be present in beer, wine and fruit juice. Especially in a beer brewing process haze formation can occur at different
10 stages. In "Enzymes in food processing" edited by T. Nagodawithana and G. Reed, 3rd edition, Academic press Inc., San Diego, Chapter V, p.448-449, it has been proposed that the haze in beer is the result of interactions between beer proteins and polyphenolic procyanidins.

[003] WO2002046381 describes a method for the prevention or reduction of haze in a beverage wherein a prolyl-specific endoprotease (also referred to as "PEP") is added to the beverage.
15 WO2002046381 explains that the activity of prolyl-specific endoproteases is dependent on the pH. It describes for example that an endoprotease is added to a beverage having a maximum prolyl specific activity at a pH which corresponds to the pH of the beverage it is added to. Since haze formation often occurs in acidic beverages such as for example beer, wine and fruit juice, prolyl-specific endoproteases having a prolyl specific activity at a pH value below 7 are preferably used. WO2002046381
20 subsequently provides several specific prolyl- specific endoproteases that have since then been very successful in reduction of haze in beverages.

[004] WO2005027953 describes the use of a proline specific endoprotease to produce food, for example beer or bread which is devoid of celiac related epitopes, preferably gluten epitopes, more preferably wheat or barley epitopes.

25 [005] In their article titled "Crystal structure and substrate recognition mechanism of the prolyl endoprotease PEP from *Aspergillus niger*", published in Biochemical and Biophysical Research Communications, Volume 591, 5 February 2022, Pages 76-81, Miyazono et al. determined the structure of PEP by X-ray crystallography to reveal the structural basis of the prolyl endoprotease activity of PEP. The PEP structure shows that PEP has a wide-open catalytic pocket compared to its
30 homologs. The characteristic catalytic pocket structure of PEP was predicted to be important for the recognition of protein substrates. The article mentions that to predict the substrate recognition mechanism of PEP, the PEP structure was superposed with the DPP7 structure in complex with inhibitor L-2,4-diaminobutylpiperidinamide (Dab-Pip). The authors believe that the Dap-Pip binding model of PEP suggested that PEP recognizes a proline residue of substrate using Glu88, Pro205,
35 Trp374 and Trp460. In passing it is mentioned that PEP shows the highest cleavage activity at pH 3.7.

[006] Miyazono et al describe that the PEP was purified from the culture filtrate of *A. niger* using DEAE-TOYOPEARL (TOSOH), Sephacryl S-100 (GE Healthcare) and pepstatin-agarose (Sigma) columns. Subsequently the concentrated PEP (30 mg/ml in 50 mM sodium acetate buffer pH 4.0 and 1 M NaCl) was crystallized using the sitting-drop vapor-diffusion method at 20 °C.

5 [007] Although the prolyl endoprotease enzyme and its activity in different applications has been studied in the past, little is known about the stages before application.

[008] For industrial applications, prolyl-specific endoprotease enzyme is often supplied in substantial quantities and needs to be transported from a first location, where it is manufactured, to a second location, where it is applied. In addition, the prolyl-specific endoprotease enzyme may require storage
10 before being used in the actual desired application process. Further, prolyl-specific endoprotease enzyme may undergo pre-processing before being applied in the desired application process.

[009] The provision of an endoprotease composition, preferably a prolyl-specific endoprotease composition, that has improved properties for transport and/or storage and/or pre-processing would be an advancement in the art.

15

Summary of the invention

[010] Inventors have now developed an endoprotease composition that has improved properties for transport and/or storage and/or pre-processing.

[011] Accordingly, in a first aspect the invention provides a composition comprising or consisting of:

20 - an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and

- a solution stabilizer and/or an osmolyte; and

- optionally a solvent.

[012] The requirements during transport and/or storage and/or pre-processing differ from the
25 requirements during process application of the endoprotease enzyme.

[013] During transport and/or storage and/or pre-processing it is key to maintain the endoprotease enzyme in solution. For example, precipitation of endoprotease enzyme in a liquid endoprotease composition during transport and/or storage and/or pre-processing may result in discrepancies once part of the composition is dosed in a predetermined amount during the desired application process.

30 After the endoprotease enzyme is dosed in the predetermined amount in the desired application process, the consequences of some precipitation are limited. For example, in a beer brewing process, any precipitation of the endoprotease enzyme will cause little issues, as it may be removed together with other precipitated matter.

[014] During transport and/or storage and/or pre-processing it is also highly desirable to limit loss of
35 the enzyme activity and/or stability. A high enzyme activity and/or stability gives the best value proposition to the customer, who can dose less of the endoprotease composition in the desired

application process. Once the endoprotease enzyme has completed its task in the desired application process, a reduction in its activity and/or stability is less of a problem and, in some cases, is desired to ensure the end-customer is supplied with a product wherein the endoprotease enzyme is substantially deactivated.

5 [015] During transport and/or storage and/or pre-processing it is further desirable to protect the endoprotease composition against spoilage by undesired micro-organisms.

[016] Accordingly, in a second aspect the invention provides a composition comprising or consisting of:

- an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and
- 10 - a further component, wherein such further component comprises or consists of 6-pentyl- α -pyrone, paracelsin or siderophores (cis-fusarinine); and
- optionally a solvent.

[017] Preferably the further component comprising or consisting of 6-pentyl- α -pyrone, paracelsin or siderophores (cis-fusarinine) is an antimicrobial component. That is, preferably the second aspect of the invention provides a composition comprising or consisting of:

- an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and
- 15 - an antimicrobial component, wherein the antimicrobial component comprises or consists of 6-pentyl- α -pyrone, paracelsin or siderophores (cis-fusarinine); and
- optionally a solvent.

Preferably the enzymatic component and the 6-pentyl- α -pyrone, paracelsin or siderophores (cis-fusarinine) are produced in the same micro-organism, preferably a bacteria or a fungus. Advantageously this can be achieved by producing both components in a *Trichoderma* species. The invention therefore also provides a microorganism, preferably a fungus, more preferably a *Trichoderma* species, most preferably a *Trichoderma reesei*, which micro-organism produces:

- 20 - an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and
- 6-pentyl- α -pyrone, paracelsin or siderophores (cis-fusarinine).

30 Preferably the microorganism, preferably a fungus, more preferably a *Trichoderma* species, most preferably a *Trichoderma reesei*, can be genetically modified to heterologous express the enzymatic component, comprising or consisting of the endoprotease, preferably a prolyl-specific endoprotease. Preferences for such endoprotease are as detailed below. The production of the endoprotease in *Trichoderma* can be carried out as described for example in WO2022/266456 or WO2020/025704.

35 Preferably the microorganism, preferably a fungus, more preferably a *Trichoderma* species, most preferably a *Trichoderma reesei*, natively produces the 6-pentyl- α -pyrone, paracelsin or siderophores

(cis-fusarinine). The above conveniently allows one to produce the 6-pentyl- α -pyrone, paracelsin or siderophores (cis-fusarinine) in-situ simultaneously with the endoprotease.

Most preferably the further, preferably antimicrobial, component is 6-pentyl- α -pyrone.

The advantage of the above composition comprising the 6-pentyl- α -pyrone, paracelsin or siderophores (cis-fusarinine) is that the composition comprising the endoprotease is less susceptible to spoilage by undesired micro-organisms than a composition without the 6-pentyl- α -pyrone, paracelsin or siderophores (cis-fusarinine).

Preferably the molar ratio of moles 6-pentyl- α -pyrone, paracelsin and/or siderophores (cis-fusarinine) present (added up all together) to moles endoprotease, is equal to or less than 1:1, more preferably equal to or less than 0.5:1, even more preferably equal to or less than 0.1:1, still more preferably equal to or less than 0.05:1 and most preferably equal to or less than 0.01:1.

Preferably the total weight amount of 6-pentyl- α -pyrone, paracelsin and/or siderophores (cis-fusarinine) present (added up all together) is equal to or more than 0.0001% w/w, more preferably equal to or more than 0.0005% w/w, still more preferably equal to or more than 0.001% w/w, even more preferably equal to or more than 0.005% w/w, yet even more preferably equal to or more than 0.01% w/w, still even more preferably equal to or more than 0.05% w/w and most preferably equal to or more than 0.1% w/w, based on the weight of the enzymatic component, more preferably based on the weight of the endoprotease. Preferably the total weight amount of 6-pentyl- α -pyrone, paracelsin and/or siderophores (cis-fusarinine) present (added up all together) is equal to or less than 50% w/w, more preferably equal to or less than 10% w/w, even more preferably equal to or less than 5% w/w and most preferably equal to or less than 1% w/w, based on the weight of the enzymatic component, more preferably based on the weight of the endoprotease.

More preferably the molar ratio of moles 6-pentyl- α -pyrone (if taken alone) to moles endoprotease, is equal to or less than 1:1, more preferably equal to or less than 0.5:1, even more preferably equal to or less than 0.1:1, still more preferably equal to or less than 0.05:1 and most preferably equal to or less than 0.01:1.

More preferably the total weight amount of 6-pentyl- α -pyrone (if taken alone) is equal to or more than 0.0001% w/w, more preferably equal to or more than 0.0005% w/w, still more preferably equal to or more than 0.001% w/w, even more preferably equal to or more than 0.005% w/w, yet even more preferably equal to or more than 0.01% w/w, still even more preferably equal to or more than 0.05% w/w and most preferably equal to or more than 0.1% w/w, based on the weight of the enzymatic component, more preferably based on the weight of the endoprotease. More preferably the total weight amount of 6-pentyl- α -pyrone (if taken alone) is equal to or less than 50% w/w, more preferably equal to or less than 10% w/w, even more preferably equal to or less than 5% w/w and most preferably equal to or less than 1% w/w, based on the weight of the enzymatic component, more preferably based on the weight of the endoprotease.

The invention conveniently also provides process for the production of a composition comprising or consisting of:

- an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and

5 - a further component, wherein such further component comprises or consists of 6-pentyl- α -pyrone, paracelsin or siderophores (cis-fusarinine); and

- optionally a solvent,

wherein the composition is produced in a micro-organism, preferably a fungus, preferably a *Trichoderma* species, preferably a *Trichoderma reesei*, for example as described in detail above.

10 Also combinations of enzymes are advantageous.

[018] In a third aspect the invention thus provides a composition comprising or consisting of:

- endoprotease, preferably prolyl-specific endoprotease; and

- one or more additional enzymes chosen from the group consisting of acetolactate decarboxylate (ALDC), aminopeptidase, trehalase and combinations thereof; and

15 - optionally a solvent.

[019] Details on all the above are provided below.

Brief description of the sequence listing

[020] This application contains a Sequence Listing in computer readable form, which is incorporated
20 herein by reference. An overview of the sequences is provided by Table 1 below.

Table 1: Overview of sequence listings:

SEQ ID No:	Enzyme/gene Name	Origin	Type
SEQ ID NO: 1	The proline-specific endoprotease described in SEQ ID NO: 5 in WO2002046381	<i>Aspergillus niger</i>	Protein
SEQ ID NO: 2	endoprotease described in SEQ ID NO:1 in WO2022/266456	<i>Aspergillus bertholletius</i>	Protein
SEQ ID NO: 3	endoprotease described in SEQ ID NO:2 in WO2022/266456	<i>Aspergillus niger</i>	Protein
SEQ ID NO: 4	endoprotease described in SEQ ID NO:3 in WO2022/266456	<i>Aspergillus transmontanensis</i>	Protein
SEQ ID NO: 5	endoprotease described in SEQ ID NO:4 in WO2022/266456	<i>Aspergillus homomorphus</i>	Protein
SEQ ID NO: 6	endoprotease described in SEQ ID NO:16 in WO2022/266456	<i>Aspergillus niger</i>	Protein

SEQ ID NO: 7	endoprotease described in SEQ ID NO:4 in WO2002046381	<i>Aspergillus niger</i>	Protein
SEQ ID NO: 8	endoprotease described in SEQ ID NO:7 in WO2002046381	<i>Aspergillus niger</i>	Protein

Preferably the endoprotease in the present invention is a polypeptide which has an amino acid sequence which has equal to or more than 40%, more preferably equal to or more than 60%, still more preferably equal to or more than 70%, even more preferably equal to or more than 80%, still even more preferably equal to or more than 90%, yet even more preferably equal to or more than 95%, and most preferably equal to or more than 99% amino acid sequence identity with the amino acid sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8, most preferably SEQ ID NO:1.

Detailed description of the invention

Definitions

[021] Unless defined otherwise or clearly indicated by context, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art.

[022] Throughout the present specification and the accompanying claims, the words "comprise" and "include" and variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. That is, these words are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows.

[023] The articles "a" and "an" are used herein to 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. When referring to a noun (e.g. a compound, an additive, etc.) in the singular, the plural is meant to be included. Thus, when referring to a specific moiety, e.g. a "strain", this means "at least one" of that strain, e.g. "at least one strain", unless specified otherwise.

[024] When referring to a compound of which several isomers exist (e.g. a D and an L enantiomer), the compound in principle includes all enantiomers, diastereomers and cis/trans isomers of that compound that may be used in the particular aspect of the invention; in particular when referring to such as compound, it includes the natural isomer(s).

[025] Unless explicitly indicated otherwise, the various embodiments of the invention described herein can be cross-combined.

[026] The terms "peptide" and "oligopeptide" are considered synonymous (as is commonly recognized) and each term can be used interchangeably as the context requires to indicate a chain of at least two amino acids coupled by peptidyl linkages. The word "polypeptide" is used herein for chains containing more than seven amino acid residues. All oligopeptide and polypeptide formulas or

sequences herein are written from left to right and in the direction from amino terminus to carboxy terminus. References to amino acid names herein refer to the amino acids as described in the handbook by Jeremy M. Berg, John L. Tymoczko and Lubert Stryer, titled Biochemistry, 6th edition, published 2007 by W.H. Freeman and Company, New York, USA, Chapter 2. The one-letter code of amino acids used herein is commonly known in the art and can be found for example in the handbook by Jeremy M. Berg, John L. Tymoczko and Lubert Stryer, titled Biochemistry, 6th edition, published 2007 by W.H. Freeman and Company, New York, USA, Chapter 2, Table 2.2.

[027] Nucleic acid sequences (i.e. polynucleotides) or proteins (i.e. polypeptides) may be native or heterologous to the genome of the host cell.

[028] "Native", "homologous" or "endogenous" with respect to a host cell, means that the nucleic acid sequence does naturally occur in the genome of the host cell or that the protein is naturally produced by that cell. The terms "native", "homologous" and "endogenous" are used interchangeable herein.

[029] As used herein, "heterologous", with respect to the host cell, may refer to a polynucleotide that does not naturally occur in that way in the genome of the host cell or that a polypeptide or protein is not naturally produced in that manner by that cell. Heterologous protein expression involves expression of a protein that is not naturally expressed in that way in the host cell. The term "heterologous expression" refers to the expression of heterologous nucleic acids in a host cell.

[030] As used herein "promoter" is a DNA sequence that directs the transcription of a (structural) gene or other (part of) nucleic acid sequence. Suitably, a promoter is located in the 5'-region of a gene, proximal to the transcriptional start site of a (structural) gene. Promoter sequences may be constitutive, inducible or repressible. In an embodiment there is no (external) inducer needed.

The enzyme component

[031] The composition according to the invention suitably comprises an enzymatic component, which enzymatic component comprises or consists of an endoprotease, preferably a prolyl-specific endoprotease.

[032] By an endoprotease is herein preferably understood an enzyme having endoprotease activity. By endoprotease activity is herein understood the ability to break a peptide bond between non-terminal amino acids within a peptide, i.e. to break a peptide bond within the molecule. The wording "endoprotease", "endoprotease enzyme", "enzyme having endoprotease activity", "protein having endoprotease activity" and "polypeptide having endoprotease activity", "endopeptidase", "endopeptidase enzyme", "enzyme having endopeptidase activity", "protein having endopeptidase activity" and "polypeptide having endopeptidase activity" are used interchangeably herein.

[033] Preferably the endoprotease is a prolyl-specific endoprotease. The term "proline-specific" and "prolyl-specific" are used interchangeably herein. In the context of the present invention, a proline-specific endoprotease is herein preferably understood as an endoprotease that cuts (hydrolyses)

proteins or peptides at places where the protein or peptide contains a proline residue in its chain. Such prolyl-specific endoproteases are sometimes also referred to as proline-specific endoproteases, prolyl-specific endopeptidases or proline-specific endopeptidases.

[034] For example, a proline-specific endoprotease is preferably used that hydrolyses the peptide
5 bond at the carboxyterminal end of proline residues. Examples of such enzymes are prolyl oligopeptidases (EC 3.4.21.26).

[035] Preferably the prolyl-specific endoprotease is an *Aspergillus niger* derived prolyl-specific endoprotease such as for example reported in J. Agric Food Chem, Vol 53 (20), 7950-7957, 2005) and proline-specific dipeptidyl peptidases such as DPP IV (EC 3.4.14.5). A proline-specific endoprotease
10 that cuts proline-residues at their NH₂-terminus is for example described in a publication in Nature of 15 January 1998, Vol.391, p.301-304.

[036] Examples of proline-specific enzymes also include the enzymes as described in WO2022/266456.

[037] In a first preferred embodiment the first enzymatic component includes one or more
15 endoprotease enzymes having an amino acid sequence that has at least 75, 80, 85, 90, 95, 98, 99 or 100% sequence identity to one or more of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 and/or SEQ ID NO: 16 as described in WO2022/266456 or an endoprotease active fragment thereof such as a mature protein. The sequence listing of these enzymes as provided with WO2022/266456 is incorporated herewith by reference. These enzymes may be produced as described in
20 WO2022/266456.

[038] In a second preferred embodiment the first enzymatic component includes one or more endoprotease enzymes having an amino acid sequence that has at least 75, 80, 85, 90, 95, 98, 99 or 100% sequence identity to one or more of SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 as described in WO2020/025704 or an endoprotease active fragment thereof such as a mature protein. The sequence
25 listing of these enzymes as provided with WO2020/025704 is incorporated herewith by reference. These enzymes may be produced as described in WO2020/025704.

[001] Other preferred prolyl-specific endoproteases include prolyl-specific endoproteases derived from *Aspergillus niger*, *Aspergillus ficuum*, *Aspergillus transmontanensis*, *Aspergillus homomorphus*, *Aspergillus pseudocaelatus*, *Aspergillus neoauricomus*, *Aspergillus albertensis*, *Aspergillus albertensis*, *Aspergillus wentii*, *Aspergillus brasiliensis*, *Aspergillus sclerotium*, *Aspergillus bertholletius*, *Aspergillus awamori*, *Aspergillus tubegensis*, *Aspergillus accretis*, *Aspergillus foretidus*, *Aspergillus nidulans*, *Aspergillus japonica*.

[002] Examples include the proline-specific proteases described in:

- the article titled "Crystal structure and substrate recognition mechanism of the prolyl endoprotease
35 PEP from *Aspergillus niger*" by Miyazono et al, published in Biochemical and Biophysical Research Communications, Volume 591, 5 February 2022, Pages 76-81; and

- in the article titled "Extracellular Prolyl Endoprotease from *Aspergillus niger* and Its Use in the Debittering of Protein Hydrolysates" by Edens et al, published in J. Agric. Food Chem. 2005, 53, 7950-7957; and

5 - In the article titled "Influence of dietary components on *Aspergillus niger* prolyl endoprotease mediated gluten degradation" by Montserrat et al., published in Biochemical and Biophysical Research Communications 591 (2022) pages 76-81, each incorporated herein by reference.

[003] Preferably the activity of the endoprotease, preferably proline-specific endoprotease, is dependent on the pH. Preferably the endoprotease, preferably proline-specific endoprotease, is an
10 endoprotease, respectively proline-specific endoprotease, with an acidic pH optimum, i.e. a pH optimum below pH 7.0. More preferably the endoprotease, preferably proline-specific endoprotease, is an endoprotease, respectively proline-specific endoprotease, having its optimum activity at a pH in the range from equal to or more than pH 1.0, more preferably from equal to or more than pH 1.2 to equal to or less than pH 6.0, more preferably to equal to or less than pH 5.0, still more preferably to equal to
15 or lower than pH 4.0, even more preferably to equal to or lower than pH 3.0 and most preferably to equal to or less than pH 2.5. Preferably the endoprotease, preferably proline-specific endoprotease, is an endoprotease, respectively proline-specific endoprotease, having its optimum activity at a pH in the range from pH 1.0 to pH 5.0, even more preferably in the range from pH 1.2 to pH 4.5.

[004] As indicated above, proline-specific proteases have for example been identified in species of
20 *Aspergillus* (EP 0 522 428), *Flavobacterium* (EP 0 967 285) and *Aeromonas* (J. Biochem.113, 790-796), *Xanthomonas* and *Bacteroides*. The proline-specific protease of the invention may be isolated from one of the above-mentioned microbial species, particularly from a species of *Aspergillus* or *Trichoderma*. Preferably, the proline-specific endoprotease is isolated from a strain of *Aspergillus niger* or *Trichoderma reesei*. More preferably, the proline-specific endoprotease is isolated from an
25 *Aspergillus niger* or *Trichoderma reesei* host engineered to overexpress a gene encoding a proline-specific endoprotease.

[005] Other hosts, such as *E. coli* can also be useful. For example, the cloning and overproduction of the *Flavobacterium* derived proline-specific endoprotease in, amongst others, *E.coli* has made certain proline-specific endoproteases available in a pure form. An example of such an overproducing
30 construct is provided in the World Journal of Microbiology & Biotechnology, Vol 11, pp 209-212.

[006] An *Aspergillus niger* host or *Aspergillus niger* or *Trichoderma reesei* host is preferably used to produce a non-recombinant self-construct utilizing *A. niger* promoters to drive the expression of a gene encoding an *A. niger* proline-specific endoprotease. That is, preferably the endoprotease, preferably proline-specific endoprotease, is a non-recombinant endoprotease, respectively non-recombinant
35 proline-specific endoprotease.

[007] Most preferably the endoprotease, preferably proline-specific endoprotease, is an endoprotease as described in EP-A-1326957, WO2005027953 or WO2002046381, which are herein incorporated by reference.

[008] Preferably the endoprotease is a prolyl-specific endoprotease selected from the group consisting of:

(a) a polypeptide which has an amino acid sequence which has equal to or more than 40%, more preferably equal to or more than 60%, still more preferably equal to or more than 70%, even more preferably equal to or more than 80%, still even more preferably equal to or more than 90%, yet even more preferably equal to or more than 95%, and most preferably equal to or more than 99% overall amino acid sequence identity with SEQ ID NO: 1, SEQ ID NO: 3, OR SEQ ID NO: 5 or a fragment thereof, each as described in WO2002046381;

(b) a polypeptide which is encoded by a polynucleotide which hybridizes with

(i) the nucleic acid sequence of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or a fragment thereof which is at least 80% or 90% identical over 60, preferably over 100 nucleotides, more preferably at least 90% identical over 200 nucleotides, each as described in WO2002046381, or

(ii) a nucleic acid sequence complementary to the nucleic acid sequence of (i).

[009] The so-called active site(s) within an enzyme are those parts of the enzyme that are responsible for interaction with the substrate. This interaction may for example comprise the binding of and/or catalysing of the reaction in the substrate. The formation and presence of such so-called active site(s) can be dependent on the enzyme's primary, secondary and/or tertiary structure. Examples of active sites include catalytic triad(s) and/or an oxyanion hole(s).

[010] Preferably the endoprotease, preferably proline-specific endoprotease, is an endoprotease, respectively proline-specific endoprotease, comprising a catalytic triad and/or an oxyanion hole. More preferably the endoprotease, preferably proline-specific endoprotease, is an endoprotease, respectively proline-specific endoprotease, comprising a catalytic triad and an oxyanion hole.

[011] Preferably the catalytic triad comprises an amino acid sequence comprising or consisting of Serine, Histidine and Aspartic acid. Such a catalytic triad is herein also referred to as a Ser/Asp/His catalytic triad. More preferably the catalytic triad comprises an amino acid sequence comprising or consisting of Ser179-His491-Asp458, where the numbers indicate the amino acid number in the amino acid sequence of the protein sequence of the proline-specific endoprotease described in SEQ ID 5 in WO2002046381, incorporated herein by reference and repeated for convenience below:

[012] MRAFSAVAAAALALSWASLAQAARPLVPKPVSRPASSKSAATTGEAYFEQLLDHHNPEKG
TFSQRYWWSTEYWGGPGSPVVLFTPGEVSADGYEGYLNETLTGVYAQEIQGAVILIEHRYWGDSS
PYEVLNAETLQYLTLDAQILDMTYFAETVKLQFDNSTRSNAQNAPWWMVGGSYSGALTAWTESVAP
GTFWAYHATSAPVEAIYDYWQYFYPIQQGMAQNCSKDVSLVAEYVDKIGKNGTAKEQQALKELFGL
GAVEHFDDFAAVLPNGPYLWQDNDNFATGYSSFFQFCDAVEGVEAGAAVTPGPEGVGLEKALANYA

NWFNSTILPDYCASYGYWTDEWSVACFDSYNASSPIYTDTSVGNVDRQWEWFLCNEPFFYWQDG
 APEGTSTIVPRLVSASYWQRQCPLYFPETNGYTYGSAKGKNAATVNSWTGGWDMTRNTTRLIWTN
 GQYDPWRDSGVSSSTFRPGGPLASTANEPVQIIPGGFHCSDLYMADYYANEGVKKVVDNEVKQIKEW
 VEEYYA (SEQ ID 5 of WO2002046381)

5 [013] In one preferred embodiment of the invention the ND1 or N δ 1 nitrogen atom of side chain of the histidine amino acid in the above catalytic triad is protonated, and the NE2 or N ϵ 2 nitrogen atom (pK_a of 6.0) of the side chain of the histidine amino acid is unprotonated. In another preferred embodiment of the invention the histidine amino acid in the above catalytic triad is deprotonated. By deprotonated histidine is herein preferably understood a histidine amino acid that has lost its proton
 10 (H⁺ ion) on its side chain NE2 or N ϵ 2 nitrogen atom.

[014] By an oxyanion hole can be understood a structural feature found in the active site of certain hydrolytic enzymes that stabilizes negatively charged transition states during catalysis, for example in esterase, lipase and peptidase enzymes. Suitably an oxyanion hole can stabilize a transition state negative charge on a deprotonated oxygen or alkoxide. Stabilising this transition state may
 15 advantageously lower the activation energy necessary for the reaction and can thus promote catalysis. The oxyanion holes in hydrolytic enzymes are typically made up from hydrogen bond-donating groups of polar or basic amino acid side chains like Tyr, Trp, Arg, Lys, Asn, Gln, Ser, Thr or also often two backbone amides that hydrogen bond with the oxygen atoms of the negatively charged transition state.

[015] However, in the composition according to the invention, the enzyme component preferably
 20 comprises or consists of an endoprotease, preferably a prolyl-specific endoprotease, comprising an oxyanion hole comprising a protonated side chain of an acidic amino acid. More preferably this oxyanion hole comprises a glutamic acid amino acid, preferably in its protonated state (Glu-H).

[016] Most preferably the enzyme component comprises or consists of an endoprotease, preferably a prolyl-specific endoprotease, comprising a Glutamic acid corresponding to Glu88 in SEQ ID 5 of
 25 WO2002046381 as listed above, preferably in a protonated state, where the number indicates the amino acid number in the amino acid sequence of the endoprotease, preferably proline-specific endoprotease in SEQ ID 5 of WO2002046381 as listed above.

Preferably, the invention provides a composition wherein the enzymatic component preferably comprises or consists of an endoprotease enzyme, preferably a prolyl-specific endoprotease enzyme,
 30 comprising an oxyanion hole comprising a glutamic acid amino acid, wherein within the majority of the endoprotease enzyme, the glutamic acid in the oxyanion hole is present in a protonated state. More preferably equal to or more than 20% mol/mol, even more preferably equal to or more than 30% mol/mol, still more preferably equal to or more than 40% mol/mol, yet more preferably equal to or more than 50% mol/mol, still even more preferably equal to or more than 60% mol/mol, yet even more
 35 preferably equal to or more than 70% mol/mol, still even more preferably equal to or more than 80% mol/mol, still even more preferably equal to or more than 90% mol/mol and most preferably equal to or

more than 95% mol/mol of the endoprotease, preferably prolyl-specific endoprotease, comprises an oxyanion hole with a glutamic acid, wherein the glutamic acid is present in a protonated state. Such can be determined in a similar manner as described by Markley et al in their article titled "Protonation-state dependence of hydrogen bond strengths and exchange rates in a serine protease catalytic triad: bovine chymotrypsinogen A", published in Biochemistry, 1996 Aug 27;35(34): pages 11092-7.

Preferably, the invention provides a composition wherein the enzymatic component preferably comprises or consists of an endoprotease enzyme, preferably a prolyl-specific endoprotease enzyme, comprising a glutamic acid corresponding to Glu88 in SEQ ID 5 of WO2002046381 as listed above, wherein within the majority of the endoprotease enzyme the glutamic acid in the Glu88 is present in a protonated state. More preferably equal to or more than 20% mol/mol, even more preferably equal to or more than 30% mol/mol, still more preferably equal to or more than 40% mol/mol, yet more preferably equal to or more than 50% mol/mol, still even more preferably equal to or more than 60% mol/mol, yet even more preferably equal to or more than 70% mol/mol, still even more preferably equal to or more than 80% mol/mol, still even more preferably equal to or more than 90% mol/mol and most preferably equal to or more than 95% mol/mol of the endoprotease of the endoprotease, preferably prolyl-specific endoprotease, comprises a Glu88, wherein the glutamic acid is present in a protonated state.

[017] Preferably, the invention provides a composition wherein the enzymatic component preferably comprises or consists of an endoprotease enzyme, preferably a prolyl-specific endoprotease enzyme, comprising a catalytic triad comprising a histidine amino acid, wherein within the majority of the endoprotease enzyme, the NE2 atom of the histidine amino acid in the catalytic triad is present in a deprotonated state. More preferably equal to or more than 20% mol/mol, even more preferably equal to or more than 30% mol/mol, still more preferably equal to or more than 40% mol/mol, yet more preferably equal to or more than 50% mol/mol, still even more preferably equal to or more than 60% mol/mol, yet even more preferably equal to or more than 70% mol/mol, still even more preferably equal to or more than 80% mol/mol, still even more preferably equal to or more than 90% mol/mol and most preferably equal to or more than 95% mol/mol of the endoprotease, preferably prolyl-specific endoprotease, comprises an catalytic triad with a histidine, wherein the NE2 atom of the histidine is present in a deprotonated state.

[018] Preferably, the invention provides a composition wherein the enzymatic component preferably comprises or consists of an endoprotease enzyme, preferably a prolyl-specific endoprotease enzyme, comprising a histidine corresponding to His491 in SEQ ID 5 of WO2002046381 as listed above, wherein within the majority of the endoprotease enzyme the NE2 atom of the histidine in the His491 is present in a deprotonated state. More preferably equal to or more than 20% mol/mol, even more preferably equal to or more than 30% mol/mol, still more preferably equal to or more than 40% mol/mol, yet more preferably equal to or more than 50% mol/mol, still even more preferably equal to or more than 60% mol/mol, yet even more preferably equal to or more than 70% mol/mol, still even more preferably equal

to or more than 80% mol/mol, still even more preferably equal to or more than 90% mol/mol and most preferably equal to or more than 95% mol/mol of the endoprotease of the endoprotease, preferably prolyl-specific endoprotease, comprises a histidine corresponding to His491 in SEQ ID 5 of WO2002046381 as listed above, wherein the NE2 atom of the histidine in His491 is present in a deprotonated state.

[019] The proline-specific protease is preferably produced in or derived from a micro-organism, preferably a bacterium or a fungus. The proline-specific protease may be a natural occurring recombinant or chemically-modified proline-specific protease enzyme. Proline-specific proteases have for example been identified in species of *Aspergillus* (EP 0 522 428), *Flavobacterium* (EP 0 967 285) and *Aeromonas* (J.Biochem.113, 790-796), *Xanthomonas* and *Bacteroides*. The proline-specific protease of the invention may be isolated from one of the above-mentioned microbial species, particularly from a species of *Aspergillus* or *Trichoderma*. Preferably, the proline-specific endoprotease is isolated from a strain of *Aspergillus niger* or *Trichoderma reesei*. More preferably, the proline-specific endoprotease is isolated from an *Aspergillus niger* or *Trichoderma reesei* host engineered to overexpress a gene encoding a proline-specific endoprotease.

[020] Other hosts, such as *E. coli* can also be useful. For example, the cloning and overproduction of the *Flavobacterium* derived proline-specific endoprotease in, amongst others, *E.coli* has made certain proline-specific endoproteases available in a pure form. An example of such an overproducing construct is provided in the World Journal of Microbiology & Biotechnology, Vol 11, pp 209-212.

[021] The enzymes can be native or heterologous to the host cell. In a preferred embodiment, the enzyme can be native to an *Aspergillus* species and be produced in such *Aspergillus* species, preferably *Aspergillus niger*. In another preferred embodiment the enzyme can be derived from an *Aspergillus* species and the enzyme may be heterologously produced in another host cell, such as a *Trichoderma reesei*.

[022] Preferred glutamine-specific and/or prolyl-specific endoproteases include glutamine-specific and/or prolyl-specific endoproteases derived from *Aspergillus niger*, *Aspergillus transmontanensis*, *Aspergillus homomorphus*, *Aspergillus pseudocaelatus*, *Aspergillus neoauricomus*, *Aspergillus albertensis*, *Aspergillus albertensis*, *Aspergillus wentii*, *Aspergillus brasiliensis*, *Aspergillus sclerotiumniger*, *Aspergillus bertholletius*, *Aspergillus awamori*, *Aspergillus tubegensis*, *Aspergillus accretis*, *Aspergillus foretidus*, *Aspergillus nidulans*, *Aspergillus japonica*. Others include *Oryzae* and *Aspergillus ficuum*, and *Trichoderma reesei*, *Fusarium graminearum*, *Penicillium chrysogenum*, *Acremonium alabramae*, *Myserio Photora Thermophil* (*Thermophilum*).

[023] Preferably the compositions as described herein are compositions, wherein the first enzymatic component is derived from and/or produced by a micro-organism, preferably a bacterium or fungus, most preferably a *Trichoderma reesei*.

[024] Especially preferred herein are glutamine-specific and/or prolyl-specific endoproteases that are derived from an *Aspergillus* species but that are heterologously produced in a *Trichoderma* species, preferably *Trichoderma reesei*.

5 [025] In another preferred embodiment, the *Aspergillus niger* host or *Aspergillus niger* or *Trichoderma reesei* host is preferably used to produce a non-recombinant self-construct utilizing *A. niger* promoters to drive the expression of a gene encoding an *A. niger* proline-specific endoprotease. That is, preferably the endoprotease, preferably proline-specific endoprotease, is a non-recombinant endoprotease, respectively non-recombinant proline-specific endoprotease.

10 [026] The composition can advantageously comprise one or more additional enzymes in addition to the endoprotease enzyme, such as for example acetolactate decarboxylate (ALDC), aminopeptidase or trehalase.

[027] The invention therefore provides in an additional aspect a composition comprising:
- endoprotease, preferably prolyl-specific endoprotease; and
- one or more additional enzymes chosen from the group consisting of a acetolactate decarboxylate
15 (ALDC), aminopeptidase or trehalase.

Preferences for the endoprotease are as described above. Preferably such a composition further comprises a solution stabilizer and/or an osmolyte and/or a solvent as described herein above and/or herein below.

20 **The composition and the pH of the composition**

[028] The composition may or may not comprise a solvent.

[029] Preferably the composition is a liquid composition. Preferably the composition comprises a solvent.

25 [030] A first preferred aspect provides a liquid composition comprising or consisting of:
- an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and
- a solution stabilizer and/or an osmolyte; and
- a solvent.

30 Preferences for the endoprotease are as described above. Preferences for the solution stabilizer and/or osmolyte are as described below.

[031] A second preferred aspect provides a liquid composition comprising or consisting of:
- an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and
- an antimicrobial component, wherein the antimicrobial component comprises or consists of 6-pentyl-
35 α -pyrone, paracelsin or siderophores (cis-fusarinine); and
- a solvent.

[032] A third preferred aspect provides a liquid composition comprising or consisting of:

- endoprotease, preferably prolyl-specific endoprotease; and
- one or more additional enzymes chosen from the group consisting of acetolactate decarboxylate (ALDC), aminopeptidase, trehalase and combinations thereof; and
- 5 - a solvent.

Preferences for the endoprotease are as described above. Preferably such a composition further comprises a solution stabilizer and/or an osmolyte and/or a solvent as described herein above and/or herein below.

[033] Preferably the solvent comprises or consists of water. In addition to the water, one or more co-solvents may or may not be present. If a co-solvent is present, the solvent can comprise water and one or more co-solvents. Preferred co-solvents include tripropylene glycol methyl ether, dipropylene glycol methyl ether, propylene glycol methyl ether, diethylene glycol butyl ether, dipropylene glycol, Methylene glycol, 1,2- propanediol, N-ethyl-2-pyrrolidinone, isopropanol, ethanol, ethyl lactate, 1,3-propanediol, and/or any combinations thereof.

[034] The solvent may or may not comprise glycerol. Preferably the solvent comprises less than 50% v/v, more preferably less than 40% v/v, still more preferably less than 30% v/v, even more preferably less than 20% v/v, and most preferably less than 10 % v/v glycerol, based on the total volume of the solvent. Most preferably the solvent does not comprise glycerol. More recently glycerol has been subject to a lot of price volatility and therefore is less preferred. The use of the solution stabilizer and/or osmolyte as described herein advantageously allows one to reduce the amount of glycerol in the solvent whilst still obtaining good stability and/or activity and/or avoiding precipitation.

[035] Preferably the composition is a liquid composition having a pH in the range from equal to or more than pH 1.0, more preferably from equal to or more than pH 1.2 to equal to or less than pH 6.0, more preferably to equal to or less than pH 5.0, still more preferably to equal to or lower than pH 4.0, even more preferably to equal to or lower than pH 3.0 and most preferably to equal to or less than pH 2.5. More preferably the composition is a liquid composition having a pH in the range from pH 1.0 to pH 5.0, even more preferably in the range from pH 1.2 to pH 4.5.

[036] The composition according to the invention can be advantageous during transport and/or storage and/or pre-processing.

[037] In an alternative embodiment the composition can be provided as a frozen or freeze dried form and be reconstituted before application by the addition of a solvent as described above during pre-processing.

[038] The invention therefore also provides a frozen or freeze-dried composition comprising or consisting of:

- 35 - an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and

- a solution stabilizer and/or an osmolyte.

Preferences are as described above and below.

[039] The invention therefore also provides a frozen or freeze-dried composition comprising or consisting of:

- 5 - an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and
- an antimicrobial component, wherein the antimicrobial component comprises or consists of 6-pentyl- α -pyrone, paracelsin or siderophores (cis-fusarinine).

Preferences are as described above and below.

10 [040] The invention therefore also provides a frozen or freeze-dried composition comprising or consisting of:

- endoprotease, preferably prolyl-specific endoprotease; and
- one or more additional enzymes chosen from the group consisting of acetolactate decarboxylate (ALDC), aminopeptidase, trehalase and combinations thereof.

15 Preferences are as described above and below.

[041] When present, the total weight of the solution stabilizer and/or osmolyte is preferably in the range from equal to or more than 5 % w/w, more preferably from equal to or more than 15 % w/w, still more preferably from equal to or more than 25 % w/w, even more preferably from equal to or more than 35 % w/w to equal to or less than 95 % w/w, more preferably to equal to or less than 75 % w/w, most
20 preferably to equal to or less than 60 % w/w, of the total weight of the one or more enzymes.

The solution stabilizer

[042] The compositions according to the above first, second, third and other aspects according to the invention may or may not comprise a solution stabilizer.

25 [043] The solution stabilizer is herein also referred to as simply "stabilizer" or "stabilization component".

[044] Where the composition comprises a solution stabilizer, the solution stabilizer preferably comprise or consist of a salt. The salt may for instance be an alkaline or alkaline-earth salt, such as a potassium chloride or a sodium chloride. These salts can also be advantageous to reduce water
30 activity. Calcium salts are less preferred and preferably the composition does not comprise any calcium salt. Where the composition comprises a salt, most preferably the salt is sodium chloride, potassium chloride or ammonium sulphate. Sodium chloride is most preferred. In a preferred embodiment the amount of alkaline or alkaline earth halogenide salt, preferably sodium chloride, in the composition is equal to or more than 5 wt%, more preferably equal to or more than 20 wt%, based on the total weight
35 of the composition.

[045] Where the composition comprises a solution stabilizer, the solution stabilizer may preferably comprise or consist of a carboxylic acid, preferably having in the range from equal to or more than 1 to equal to or less than 10 carbon atoms, preferably in the range from equal to or more than 1 to equal to or less than 7 carbon atoms, and/or any ester and/or any salt thereof. Preferred are formic acid, acetic acid, diacetic acid, ascorbic acid, lactic acid, citric acid, sorbic acid, propionic acid, oxalic acid, malic acid and/or fumaric acid, and/or any ester thereof and/or any salt thereof. Preferred salts are for example potassium formate, potassium acetate, potassium diacetate, potassium ascorbate, potassium lactate, potassium citrate, potassium sorbate, sodium formate, sodium acetate, sodium diacetate, sodium ascorbate, sodium lactate, sodium citrate, sodium sorbate and/or combinations thereof. Of these, potassium sorbate, sodium sorbate and combinations thereof are highly preferred.

[046] Where the composition comprises a solution stabilizer, the solution stabilizer may preferably comprise or consist of a glyceryl monoacetate, glyceryl diacetate, glyceryl triacetate, glyceryl monopropionate, glyceryl dipropionate, glyceryl tripropionate, glyceryl monobutanoate, glyceryl dibutanoate, glyceryl tributanoate, glyceryl monolactate, glyceryl dilactate, glyceryl trilactate.

[047] Combinations of the above different groups are also possible.

[048] Preferably the solution stabilizer comprises or consists of a carboxylic acid and/or dicarboxylic acid having in the range from equal to or more than 1 to equal to or less than 10 carbon atoms, preferably in the range from equal to or more than 2 to equal to or less than 7 carbon atoms, even more preferably in the range from equal to or more than 2 to equal to or less than 6 carbon atoms, and most preferably in the range from equal to or more than 2 to equal to or less than 6 carbon atoms and/or any ester and/or any salt thereof.

[049] Preferred examples of suitable solution stabilizers are those comprising or consisting of formic acid, acetic acid, ascorbic acid, acetoacetic acid, lactic acid, citric acid, sorbic acid, propionic acid, butanoic acid, oxalic acid, malic acid, succinic acid and/or fumaric acid, and/or any ester thereof and/or any salt thereof.

[050] The solution stabilizer may or may not comprise benzoic acid and/or any ester thereof and/or any salt thereof. However, preferably the solution stabilizer does not comprise or consist of benzoic acid and/or any ester thereof and/or any salt thereof. Benzoates such as sodium benzoate and potassium benzoate are known food preservatives that inhibit the growth of mold, yeast and some bacteria to extend shelf life. However, their presence in food is a cause for concern for many consumers. While benzoates are allowed by regulatory authorities, many people are beginning to ask if these chemical preservatives really are beneficial to their health and are seeking to eliminate them from their diets. Beverages therefore prefer to provide benzoate-free products.

[051] Hence, preferably the carboxylic acid and/or dicarboxylic acid is a non-aromatic one and preferably the stabilizer component does not comprise or consist of benzoic acid and/or any ester thereof and/or any salt thereof.

[052] The solution stabilizer may comprise or consist of a carboxylic acid and/or dicarboxylic acid, it may comprise or consist of a carboxylic acid salt and/or dicarboxylic acid salt, or it may comprise or consist of a carboxylic acid ester and/or dicarboxylic acid ester.

5 [053] In one preferred embodiment the solution stabilizer comprises or consists of an ester of a carboxylic acid and a sugar alcohol or glycerol and/or an ester of a dicarboxylic acid and a sugar alcohol or glycerol. Preferred examples of such include glyceryl monoacetate, glyceryl diacetate, glyceryl triacetate, glyceryl monolactate, glyceryl dilactate and glyceryl trilactate.

[054] In yet another preferred embodiment the solution stabilizer comprises a source of peroxide, preferably hydrogen peroxide.

10

The Osmolyte

[055] The compositions according to the above first, second, third and other aspects according to the invention may or may not comprise an osmolyte. The osmolyte may assist in reducing water activity.

15 [056] Where the composition comprises an osmolyte, the osmolyte preferably comprises or consists of a monosaccharide, a disaccharide and/or an alcohol-derivative or chloride-derivative of such a monosaccharide or disaccharide. Preferably the osmolyte comprises or consists of sorbitol, mannitol, inositol, trehalose, sucrose, mannose and/or sucralose. Of these, sorbitol, mannitol and inositol are highly preferred.

[057] Hence in a preferred embodiment the invention provides a composition comprising or
20 consisting of:

- an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and
- a solution stabilizer and an osmolyte; and
- optionally a solvent.

25 wherein the solution stabilizer is a salt of sorbic acid, preferably sodium sorbate, potassium sorbate or a combination thereof; and

wherein the osmolyte is an alcohol-derivative of a monosaccharide or disaccharide, preferably sorbitol, mannitol, inositol or a combination thereof.

[058] An especially preferred composition is a composition comprising or consisting of:

- 30
- an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and
 - a solution stabilizer wherein the solution stabilizer is sodium sorbate, potassium sorbate or a combination thereof, preferably potassium sorbate; and
 - an osmolyte, wherein the osmolyte is sorbitol, mannitol, inositol or a combination thereof, preferably
35 sorbitol.

[059] Sorbitol can be a liquid. Hence, if the osmolyte is sorbitol, an additional solvent may

advantageously not be needed and may or may not be present.

[060] Hence in a preferred embodiment the invention provides a composition comprising or consisting of:

- an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and
- a solution stabilizer, wherein the solution stabilizer comprises or consists of formic acid, acetic acid, diacetic acid, ascorbic acid, lactic acid, citric acid, sorbic acid, propionic acid, oxalic acid, malic acid and/or fumaric acid, and/or any ester thereof and/or any salt thereof, and preferably the solution stabilizer comprises or consists of potassium sorbate and/or sodium sorbate; and
- an osmolyte wherein the osmolyte is sorbitol; and
- optionally a solvent.

In a preferred embodiment the solvent may be absent as the sorbitol may act itself as solvent. Alternatively the sorbitol could be present in combination with water or another polyol. In such case, the solvent may suitably be water or glycerol.

[061] In a most preferred embodiment, the composition comprises or consists of:

- an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and
- potassium sorbate and/or sodium sorbate; and
- sorbitol.

Additional components

[062] A possible additional component is glycerol. Also glycerol can be advantageous to reduce water activity. Unfortunately, the price of glycerol has steeply increased. In a preferred embodiment the amount of glycerol in the composition is therefore equal to or less than 50 wt%, more preferably equal to or less than 40 wt%, still more preferably equal to or less than 30 wt%, even more preferably equal to or less than 20 wt% and most preferably equal to or less than 10 wt% glycerol, based on the total weight of the composition. As described above, the composition preferably comprises a limited amount or no glycerol.

[063] A preferred potential additional component comprises or consists of an oligosaccharide. Most preferably such an oligosaccharide excipient is selected from the group consisting of maltodextrin, xylan, mannan, fucoidan, galactomannan, chitosan, raffinose, stachyose, pectin, insulin, levan, graminan, amylopectin, and mixtures thereof.

CLAIMS

1. A composition comprising or consisting of:
 - an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and
 - a solution stabilizer and/or an osmolyte; and
 - optionally a solvent.
2. A composition comprising or consisting of:
 - an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and
 - an antimicrobial component, wherein the antimicrobial component comprises or consists of 6-pentyl- α -pyrone, paracelsin or siderophores (cis-fusarinine); and
 - optionally a solvent.
3. A composition comprising or consisting of:
 - endoprotease, preferably prolyl-specific endoprotease; and
 - one or more additional enzymes chosen from the group consisting of acetolactate decarboxylate (ALDC), aminopeptidase, trehalase and combinations thereof; and
 - optionally a solvent.
4. The composition to anyone of the preceding claims, wherein the composition comprises a solution stabilizer, wherein the solution stabilizer comprises or consists of a salt, preferably chosen from the group consisting of sodium chloride, potassium chloride and ammonium sulphate.
5. The composition according to anyone of the preceding claims, wherein the composition comprises a solution stabilizer, wherein the solution stabilizer comprises or consists of a source of peroxide or a carboxylic acid, preferably having in the range from equal to or more than 1 to equal to or less than 10 carbon atoms, preferably in the range from equal to or more than 1 to equal to or less than 7 carbon atoms, and/or any ester and/or any salt thereof.
6. The composition according to anyone of the preceding claims, wherein the composition comprises a solution stabilizer, wherein the solution stabilizer comprises or consists of formic acid, acetic acid, diacetic acid, ascorbic acid, lactic acid, citric acid, propionic acid, oxalic acid, malic acid and/or fumaric acid, and/or any ester thereof and/or any salt thereof.

7. The composition according to anyone of the preceding claims, wherein the composition comprises a solution stabilizer, wherein the solution stabilizer comprises or consists of a glyceryl monoacetate, glyceryl diacetate, glyceryl triacetate, glyceryl monopropionate, glyceryl dipropionate, glyceryl tripropionate, glyceryl monobutanoate, glyceryl dibutanoate, glyceryl tributanoate, glyceryl monolactate, glyceryl dilactate, glyceryl trilactate.
8. The composition according to anyone of the preceding claims, wherein the composition comprises an osmolyte, wherein the osmolyte comprises or consists of a monosaccharide, a disaccharide and/or an alcohol-derivative or chloride-derivative of such a monosaccharide or disaccharide.
9. The composition according to anyone of the preceding claims, wherein the composition comprises an osmolyte, wherein the osmolyte comprises or consists of sorbitol, mannitol, inositol, trehalose, sucrose, mannose and/or sucrose.
10. The composition according to anyone of the preceding claims, wherein the composition does not comprise glycerol.
11. The composition according to anyone of the preceding claims, wherein the composition does not comprise benzoic acid or a derivative thereof.
12. The composition according to anyone of the preceding claims, wherein the composition further comprises an additional stabilizer, preferably chosen from the group consisting of maltodextrin, xylan, mannan, fucoidan, galactomannan, chitosan, raffinose, stachyose, pectin, inulin, levan, graminan, amylopectin, and mixtures thereof.
13. The composition according to anyone of the preceding claims, wherein the composition comprises water and optionally one or more co-solvents.
14. The composition according to anyone of the preceding claims, wherein a cosolvent is present and the cosolvent comprises or consists of tripropylene glycol methyl ether, dipropylene glycol methyl ether, propylene glycol methyl ether, diethylene glycol butyl ether, dipropylene glycol, Methylene glycol, 1,2- propanediol, N-ethyl-2-pyrrolidinone, isopropanol, ethanol, ethyl lactate, 1,3-propanediol, and/or any combination thereof.

15. The composition according to anyone of the preceding claims, wherein the composition has a pH value in the range from equal to more than 1.0 to equal to or less than 6.0.

5 16. The composition according to anyone of claims 1-3, 6, 8-15, wherein the composition comprises or consists of:

- an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and

10 - a solution stabilizer, wherein the solution stabilizer comprises or consists of formic acid, acetic acid, diacetic acid, ascorbic acid, lactic acid, citric acid, sorbic acid, propionic acid, oxalic acid, malic acid and/or fumaric acid, and/or any ester thereof and/or any salt thereof, and preferably the solution stabilizer comprises or consists of potassium sorbate and/or sodium sorbate; and

- an osmolyte, wherein the osmolyte is sorbitol; and

- optionally a solvent.

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17. The composition according to claim 16, wherein the composition comprises or consists of:

- an enzymatic component, comprising or consisting of an endoprotease, preferably a prolyl-specific endoprotease; and

20 - a solution stabilizer, wherein the solution stabilizer comprises or consists of potassium sorbate and/or sodium sorbate; and

- an osmolyte, wherein the osmolyte is sorbitol.

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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. ☒ 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:

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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
2 (completely); 1, 4-17 (partially)
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims;; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☒ No protest accompanied the payment of additional search fees.

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A. CLASSIFICATION OF SUBJECT MATTER					
INV.	C12N9/62	C12N9/96	C12P21/06	A23J3/34	A23L2/84
	A23L33/18	C12C11/00	C12C5/00		
ADD.					
According to International Patent Classification (IPC) or to both national classification and IPC					
B. FIELDS SEARCHED					
Minimum documentation searched (classification system followed by classification symbols)					
C12N C12P A23L A23J C12C					
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, BIOSIS, EMBASE, WPI Data					
C. DOCUMENTS CONSIDERED TO BE RELEVANT					
Category*	Citation of document, with indication, where appropriate, of the relevant passages				Relevant to claim No.
X	CN 104 560 933 A (UNIV JIANGNAN) 29 April 2015 (2015-04-29)				1,4, 8-11,13, 15
Y	paragraphs [0006], [0010] - [0012], [0015] Embodiments: 2-8 claims 1-8				12,14
Y	CN 106 222 149 A (DU PONT) 14 December 2016 (2016-12-14) *** Y Inventions 1 + 5 *** paragraph [0247] claims 1-5				1,2,4-17
Y	EP 1 788 081 A1 (ASAHI KASEI PHARMA CORP [JP]) 23 May 2007 (2007-05-23) *** Y Inventions 1 and 5 *** paragraphs [0057], [0060], [0061]				1,2,4-17
	- / - -				
☒ Further documents are listed in the continuation of Box C.					
☒ See patent family annex.					
* Special categories of cited documents :					
"A" document defining the general state of the art which is not considered to be of particular relevance			"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		
"E" earlier application or patent but published on or after the international filing date			"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		
"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)			"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		
"O" document referring to an oral disclosure, use, exhibition or other means			"&" document member of the same patent family		
"P" document published prior to the international filing date but later than the priority date claimed					
Date of the actual completion of the international search			Date of mailing of the international search report		
2 August 2024			20/08/2024		
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016			Authorized officer Tudor, Mark		

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International application No

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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.
Y	WO 98/00530 A1 (MOLECULAR BIOLOGY RESOURCES [US]) 8 January 1998 (1998-01-08) *** Y against invention 8 *** page 8, line 13 - page 9, line 5 claims 40-63 -----	14
A	JIANG BIN ET AL: "Effective separation of prolyl endopeptidase from Aspergillus Niger by aqueous two phase system and its characterization and application", INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES, ELSEVIER BV, NL, vol. 169, 19 December 2020 (2020-12-19), pages 384-395, XP086457235, ISSN: 0141-8130, DOI: 10.1016/J.IJBIOMAC.2020.12.120 [retrieved on 2020-12-19] ***Stays an A-doc*** abstract page 384 - page 395 -----	1,4,8-15
X	WO 2012/174127 A1 (ALVINE PHARMACEUTICALS INC [US]; SIEGEL MATTHEW JOHN [US]) 20 December 2012 (2012-12-20) ***Invention 5 = carboxylics*** paragraph [0042] claims 1,8,9 -----	1,5,6, 10,11, 13,15
A	WO 02/46381 A2 (DSM NV [NL]; LOPEZ MICHEL [FR]; EDENS LUPPO [NL]) 13 June 2002 (2002-06-13) cited in the application ***Stays an A-doc*** claims 1-37 -----	1,4,8-15
X	US 2019/358335 A1 (RUSSELL ALAN J [US] ET AL) 28 November 2019 (2019-11-28) ***Invention 8 = PEP + sorbitol*** paragraphs [0106], [0122], [0123], [0200] -----	1,8-11, 13,15
X	WO 2015/185593 A1 (DSM IP ASSETS BV [NL]) 10 December 2015 (2015-12-10) invention 8 = PEP + sorbitol page 24, line 20 - line 22 -----	1,8-11, 13,15
X	WO 2007/088062 A2 (DSM IP ASSETS BV [NL]; EDENS LUPPO [NL]; DE DECKERE EMILE [NL]) 9 August 2007 (2007-08-09) Inventions 5 and 8 = PEP lactic acid (shelf stability) and PEP + sorbitol examples 1,5 -----	1,5,6, 8-13, 15-17 2,14
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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.
Y	CUTLER HORACE G. ET AL: "6-Pentyl-[alpha]-pyrone from Trichoderma harzianum: Its Plant Growth Inhibitory and Antimicrobial Properties", AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 50, no. 11, 1 November 1986 (1986-11-01), pages 2943-2945, XP093192264, JP ISSN: 0002-1369, DOI: 10.1080/00021369.1986.10867860 Note page 2943 - page 2945 -----	2,4-15
Y	RITIENI A. ET AL: "Paracelsin E, a New Peptaibol from Trichoderma saturnisporum", JOURNAL OF NATURAL PRODUCTS, vol. 58, no. 11, 1 November 1995 (1995-11-01), pages 1745-1748, XP093192267, US ISSN: 0163-3864, DOI: 10.1021/np50125a017 abstract page 1745 - page 1748 -----	2,4-15
Y	RENSHAW ET AL: "Fungal siderophores: structures, functions and applications", MYCOLOGICAL RESEARCH, ELSEVIER, GB, vol. 106, no. 10, 1 October 2002 (2002-10-01), pages 1123-1142, XP022443299, ISSN: 0953-7562, DOI: 10.1017/S0953756202006548 abstract page 1123 - page 1142 -----	2,4-15

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 4, 8-15(all partially)

a composition comprising a prolyl-specific endoprotease and a solution stabilizer, wherein said solution stabilizer is sodium chloride; and subject-matter related thereto.

2. claims: 1, 4, 8-15(all partially)

a composition comprising a prolyl-specific endoprotease and a solution stabilizer, wherein said solution stabilizer is potassium chloride; and subject-matter related thereto.

3. claims: 1, 4, 8-15(all partially)

a composition comprising a prolyl-specific endoprotease and a solution stabilizer, wherein said solution stabilizer is ammonium sulphate; and subject-matter related thereto.

4. claims: 1, 5, 8-15(all partially)

a composition comprising a prolyl-specific endoprotease and a solution stabilizer, wherein said solution stabilizer is a source of peroxide; and subject-matter related thereto.

5. claims: 1, 5, 6, 8-17(all partially)

a composition comprising a prolyl-specific endoprotease and a solution stabilizer, wherein said solution stabilizer is a carboxylic acid; and subject-matter related thereto.

6. claims: 1, 7-15(all partially)

a composition comprising a prolyl-specific endoprotease and a solution stabilizer, wherein said solution stabilizer is a glycerly compound; and subject-matter related thereto.

7. claims: 1, 8-15(all partially)

a composition comprising a prolyl-specific endoprotease and an osmolyte, wherein said osmolyte is a saccharide; and subject-matter related thereto.

8. claims: 1, 8-17(all partially)

a composition comprising a prolyl-specific endoprotease and

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an osmolyte, wherein said osmolyte is sorbitol; and
subject-matter related thereto.

9. claims: 1, 8-15(all partially)

a composition comprising a prolyl-specific endoprotease and
an osmolyte, wherein said osmolyte is mannitol; and
subject-matter related thereto.

10. claims: 1, 8-15(all partially)

a composition comprising a prolyl-specific endoprotease and
an osmolyte, wherein said osmolyte is inositol; and
subject-matter related thereto.

11. claims: 2(completely); 4-15(partially)

a composition comprising a prolyl-specific endoprotease and
an antimicrobial component, wherein the antimicrobial
component comprises or consists of 6-pentyl-a-pyrone,
paracelsin or siderophores (cis-fusarinine); and
subject-matter related thereto.

12. claims: 3(completely); 4-15(partially)

a composition comprising a prolyl-specific endoprotease and
one or more additional enzymes chosen from the group
consisting of acetolactate decarboxylate (ALDC),
aminopeptidase, trehalase; and subject-matter related
thereto.
