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- (71) **Applicant:** NOVOZYMES A/S [DK/DK]; Krogshoejvej
36 DK-2880 Bagsvaerd (DK).
- (72) **Inventor; and**
- (71) **Applicant :** WANG, Yongjiang, [CN/CN]; 14 Xinx
Road, Shangdi Zone, Haidian District, Beijing 100085
(CN).
- (74) **Agent:** SHANGCHENG & PARTNERS; 18th Floor,
Ever Bright World Center Building, No. 28 West Ping An
Li Avenue, Xicheng District, Beijing 100034 (CN).
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(54) **Title:** METHOD FOR PRODUCING A YEAST EXTRACT

(57) **Abstract:** A method for producing a yeast extract comprises steps: a) adding an enzyme composition to yeast comprising pro-
tein; b) incubating so as to hydrolyze the protein, wherein the enzyme composition comprises papain and a second protease.



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METHOD FOR PRODUCING A YEAST EXTRACT

TECHNICAL FIELD

5 The present invention relates to a method for producing a yeast extract using an enzyme composition comprising proteases.

REFERENCE TO A SEQUENCE LISTING

10 This application contains a Sequence Listing in computer readable form. The computer readable form is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Yeast extracts are widely used, *e.g.*, for flavour in the food industries, in microorganism fermentation media, and as health foods. The production of yeast extract is described in literature, see *e.g.* Kelly, M. (1982) Yeast Extract (In: Industrial Enzymology, Godfrey, T. ed.) or Chae, H. J. et al. (2001), Bioresource Technology 76, 253-258. It is typically manufactured by breaking down the yeast by acid hydrolysis or mechanical or chemical disruption of the cells followed by autolysis with endogenous enzymes to degrade the macromolecular structures of the yeast, in particular the proteins, into the maximum amount of soluble material. Possibly, exogenous enzymes, including proteases, such as papain, are added to augment the effect of the yeast's own enzymes. After the enzymatic hydrolysis, the yeast extract is separated from the cell debris and possibly pasteurized and concentrated.

20 GB2075054A disclosed a process for the production of a yeast extract, wherein papain is used as a proteolytic enzyme. WO 2008/077890 disclosed a method for producing a yeast extract using a protease derived from *Nocardiopsis sp.* NRRL 18262. But there is still a desire to produce yeast extract with high extract yield.

SUMMARY OF THE INVENTION

30 The object of the present invention is to provide an improved process to produce yeast extract and its enzyme composition. The inventors surprisingly found that use of an enzyme composition comprising papain and a second protease can achieve synergistic effect, for example, such enzyme composition can be used to effectively improve the yield of the yeast extract, meanwhile, the turbidity and taste of yeast extract prepared by the invention are almost the same as the yeast extract prepared by using papain as exogenous protease only.

35 In one aspect, the present invention relates to a method for producing a yeast

extract, comprising:

- a) adding an enzyme composition to yeast comprising protein; and
- b) incubating so as to hydrolyse the protein,

wherein the enzyme composition comprises papain and a second protease, and the second protease is selected from the group consisting of:

- (i) a polypeptide comprising or consisting of amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353;
- (ii) a polypeptide comprising or consisting of an amino acid sequence having at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, 97%, 98%, 99% or 100% identity to amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353;
- (iii) a variant comprising a substitution, deletion, and/or insertion of one or more (*e.g.*, several) amino acids of amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353; and
- (iv) a fragment of the polypeptide of (i), (ii), or (iii) that has protease activity.

In a preferred embodiment, the second protease is derived from genus *Thermoascus*, preferably from *Thermoascus aurantiacus*, most preferably from *Thermoascus aurantiacus* CGMCC No. 0670.

In a preferred embodiment, the yeast is *Saccharomyces*, *Kluveromyces* or *Candida*, preferably the yeast is *Saccharomyces cerevisiae* or *Saccharomyces uvarum*.

In a preferred embodiment, the papain is present at an amount of 0.0125~12.5 mg enzyme protein per g yeast dry matter, preferably at an amount of 0.025~5 mg enzyme protein per g yeast dry matter, preferably at an amount of 0.125~2.5 mg enzyme protein per g yeast dry matter, preferably at amount of 0.25~1.5 mg enzyme protein per g yeast dry matter, and most preferably at an amount of 0.5~1 mg per g yeast dry matter.

In a preferred embodiment, based on the protease activity the papain is present at an amount of 30~30000 U per g yeast dry matter, preferably at an amount of 60~12000 U per g yeast dry matter, preferably at an amount of 300~6000 U per g yeast dry matter, preferably at amount of 600~3600 U per g yeast dry matter, and most preferably at an amount of 1200~2400 U per g yeast dry matter.

In a preferred embodiment, the second protease is present at an amount of 0.002-2 mg enzyme protein per g yeast dry matter, preferably at an amount of 0.004~0.4 mg enzyme protein per g yeast dry matter, preferably at amount of 0.02~0.2 mg enzyme protein per g yeast dry matter, and most preferably at an amount of 0.04~0.08 mg enzyme protein per g yeast dry matter.

In a preferred embodiment, based on the protease activity the second protease is present at an amount of 0.001~1 FLPU-A per g yeast dry matter, preferably at an amount of 0.002~0.2 FLPU-A per g yeast dry matter, preferably at an amount of 0.01~0.1 FLPU-A per g yeast dry matter, and most preferably at an amount of 0.02~0.04 FLPU-A per g yeast dry matter.

In other aspects, the present invention relates to an enzyme composition comprising papain and a second protease, and relates to use of the enzyme composition to produce a yeast extract, and relates to the yeast extract produced by the present invention.

DETAILED DISCLOSURE OF THE INVENTION

Protease

The term "protease" is defined herein as an enzyme that hydrolyses peptide bonds. It includes any enzyme belonging to the EC 3.4 enzyme group (including each of the thirteen subclasses thereof). The EC number refers to Enzyme Nomenclature 1992 from NC-IUBMB, Academic Press, San Diego, California, including supplements 1-5 published in Eur. J. Biochem. 1994, 223, 1-5; Eur. J. Biochem. 1995, 232, 1-6; Eur. J. Biochem. 1996, 237, 1-5; Eur. J. Biochem. 1997, 250, 1-6; and Eur. J. Biochem. 1999, 264, 610-650; respectively. The nomenclature is regularly supplemented and updated; see e.g. the World Wide Web (WWW) at <http://www.chem.qmw.ac.uk/iubmb/enzyme/index.html>). Proteases are also called, e.g., peptidases, proteinases, peptide hydrolases, or proteolytic enzymes.

The proteases for use according to the invention are of the endo-type that act internally in polypeptide chains (endopeptidases). There are no limitations on the origin of the protease for use according to the invention. Thus, the term protease includes not only natural or wild-type proteases, but also any mutants, variants, fragments etc. thereof exhibiting protease activity, as well as synthetic proteases, such as shuffled proteases, and consensus proteases. Such genetically engineered proteases can be prepared, which is generally known in the art, e.g., by site-directed mutagenesis, by PCR (using a PCR fragment containing the desired mutation as one of the primers in the PCR reactions), or by random mutagenesis. The preparation of consensus proteins is described in e.g. EP 897985. Examples of protease variants, as used in the present context, are proteases in which one or more amino acids have been deleted, inserted or substituted with other amino acids.

Papain, also known as papaya proteinase I, is a cysteine protease (EC 3.4.22.2) enzyme present in papaya (*Carica papaya*) and mountain papaya (*Vasconcellea cundinamaricensis*). Papain consists of a single polypeptide chain with three disulfide bridges and a sulfhydryl group necessary for activity of the enzyme. Papain digests most protein substrates more extensively than the pancreatic proteases. Papain exhibits broad specificity, cleaving peptide bonds of basic amino acids, leucine, or glycine.

Proteases are classified on the basis of their catalytic mechanism into the following groups: Serine proteases (S), Cysteine proteases (C), Aspartic proteases (A), Metalloproteases (M), and Unknown, or as yet unclassified, proteases (U), see Handbook of Proteolytic Enzymes, A.J.Barrett, N.D.Rawlings, J.F.Woessner (eds), Academic Press (1998),
 5 in particular the general introduction part.

In some preferred embodiments, the second proteases of the invention are selected from the group consisting of:

- (a) proteases belonging to the EC 3.4.24 metalloendopeptidases;
- (b) metalloproteases belonging to the M group of the above Handbook;
- 10 (c) metalloproteases not yet assigned to clans (designation: Clan MX), or belonging to either one of clans MA, MB, MC, MD, ME, MF, MG, MH (as defined at pp. 989-991 of the above Handbook);
- (d) other families of metalloproteases (as defined at pp. 1448-1452 of the above Handbook);
- 15 (e) metalloproteases with a HEXXH motif;
- (f) metalloproteases with an HEFTH motif;
- (g) metalloproteases belonging to either one of families M3, M26, M27, M32, M34, M35, M36, M41, M43, or M47 (as defined at pp. 1448-1452 of the above Handbook); and
- (h) metalloproteases belonging to family M35 (as defined at pp. 1492-1495 of the above
 20 Handbook).

In other particular embodiments, metalloproteases are hydrolases in which the nucleophilic attack on a peptide bond is mediated by a water molecule, the water molecule being activated by a divalent metal cation. Examples of divalent cations are zinc, cobalt or manganese. The metal ion may be held in place by amino acid ligands. The number of
 25 ligands may be five, four, three, two, one or zero. In a particular embodiment the number is two or three, preferably three.

For determining whether a given protease is a metalloprotease or not, reference is made to the above Handbook and the principles indicated therein. Such determination can be carried out for all types of proteases, be it naturally occurring or wild-type proteases; or
 30 genetically engineered or synthetic proteases.

The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "sequence identity".

For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch,
 35 1970, J. Mol. Biol. 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends Genet. 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of

BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

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

An exact match occurs when the two sequences have identical amino acid residues in the same positions of the overlap. The length of a sequence is the number of amino acid residues in the sequence (e.g., the length of amino acids 1-177 of SEQ ID NO: 1 herein is 177).

The term "variant" means a polypeptide having protease activity comprising an alteration, *i.e.*, a substitution, insertion, and/or deletion at one or more (e.g., several) positions. A substitution means replacement of the amino acid occupying a position with a different amino acid; a deletion means removal of the amino acid occupying a position; and an insertion means adding an amino acid adjacent to and immediately following the amino acid occupying a position. When variants are described, the nomenclature described below is adapted for ease of reference. The accepted IUPAC single letter or three letter amino acid abbreviation is employed.

In a preferred embodiment, the second protease is selected from the group consisting of:

- (i) a polypeptide comprising or consisting of amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353;
- (ii) a polypeptide comprising or consisting of an amino acid sequence having at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, 97%, 98%, 99% or 100% identity to amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353;
- (iii) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., several) amino acids of amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353; and
- (iv) a fragment of the polypeptide of (i), (ii), or (iii) that has protease activity.

In a particular embodiment, the homologous polypeptides have an amino acid sequence that differs by forty, thirty-five, thirty, twenty-five, twenty, or fifteen amino acids. In another embodiment, the homologous polypeptides have an amino acid sequence that differs by ten, nine, eight, seven, six, or five amino acids. In another particular embodiment, the homologous polypeptides differ by four, three, two amino acids, or one amino acid from amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353.

In a preferred embodiment, a fragment of amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353, is a polypeptide having one or more amino acids deleted from the amino and/or carboxyl terminus of these amino acid sequences. In one embodiment a fragment contains at least 75 amino acid residues, or at least 100 amino acid residues, or at least 125 amino acid residues, or at least 150 amino acid residues, or at least 160 amino acid residues, or at least 165 amino acid residues, or at least 170 amino acid residues, or at least 175 amino acid residues.

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

The amino acid sequences of the variant polypeptides may differ from amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353 by an insertion or deletion of one or more amino acid residues and/or a substitution of one or more amino acid residues by different amino acid residues. Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions which do not generally alter the specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, In, The Proteins, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly as well as these in reverses.

In one embodiment, the second protease for use according to the invention is a fungal protease, the term fungal indicating that the protease is derived from, or originates from, a fungal, or is an analogue, a fragment, a variant, a mutant, or a synthetic protease derived from a fungal. It may be produced or expressed in the original wild-type fungal strain, in another microbial strain, or in a plant; i.e., the term covers the expression of wild-type, naturally occurring proteases, as well as expression in any host of recombinant, genetically

engineered or synthetic proteases.

In one embodiment, the second protease is a yeast polypeptide such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide; or more preferably a fungal polypeptide such as an *Acremonium*, *Aspergillus*, *Aureobasidium*,
 5 *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*,
Neocallimastix, *Neurospora*, *Paecilomyces*, *Penicillium*, *Piromyces*, *Schizophyllum*,
Talaromyces, *Thermoascus*, *Thielavia*, *Tolypocladium*, or *Trichoderma* polypeptide. In another embodiment, the polypeptide is an *Aspergillus aculeatus*, *Aspergillus awamori*,
Aspergillus foetidus, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus*
 10 *oryzae*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium*
culmorum, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium*
negundi, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium*
sambucinum, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*,
Fusarium torulosum, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*,
 15 *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*,
Penicillium purpurogenum, *Thermoascus aurantiacus*, *Trichoderma harzianum*, *Trichoderma*
koningii, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride*
 polypeptide.

In a preferred embodiment, the second protease has been previously described, for
 20 example, the protease described in WO 2003/048353 or WO2011/072191, the contents of
 WO 2003/048353 or WO2011/072191 are cited herein as a part of the present invention.

In a preferred embodiment, the second protease is derived from a fungus of the
 genus *Thermoascus*, for example the species *Thermoascus aurantiacus*, such as the strain
Thermoascus aurantiacus CGMCC No. 0670, e.g., a polypeptide with the amino acid
 25 sequence of amino acids -178 to 177, -159 to 177, or +1 to 177 of SEQ ID NO: 2 in WO
 2003/048353, or amino acids 1-177 of SEQ ID NO: 1 herein.

The *Thermoascus aurantiacus* protease, the mature polypeptide of which comprises
 or consists of amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID
 NO: 2 in WO 2003/048353 is one example of a polypeptide of the invention.

In a preferred embodiment, the second protease of the invention is a variant of the
 protease of amino acids 1 to 177 of SEQ ID NO: 2, viz. it is not identical to amino acids 1 to
 177 of SEQ ID NO: 2, as it comprises at least one modification as compared to amino acids 1
 to 177 of SEQ ID NO: 2. The variant of the second protease of amino acids 1 to 177 of SEQ
 ID NO: 1 herein, which consists of the following modification:
 35 A27K/D79L/Y82F/S87G/D104P/A112P/A126V/D142L, is another example of a second
 protease of the invention. The position numbers refer to the position numbering of amino
 acids 1 to 177 of SEQ ID NO: 2, as described in the section "Position Numbering" in
 WO2011/072191.

It will be understood that for the aforementioned species, the invention encompasses both the perfect and imperfect states, and other taxonomic equivalents, *e.g.*, anamorphs, regardless of the species name by which they are known. Those skilled in the art will readily recognize the identity of appropriate equivalents.

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

In the process of the invention the protease may be purified. The term "purified" as used herein covers enzyme protein essentially free from components from the organism from which it is derived. The term "purified" also covers enzyme protein free from components from the native organism from which it is obtained, this is also termed "essentially pure" enzyme and may be particularly relevant for enzymes which are naturally occurring and which have not been modified genetically, such as by deletion, substitution or insertion of one or more amino acid residues.

Accordingly, a protease may be purified, *viz.* only minor amounts of other proteins being present. The expression "other proteins" relates in particular to other enzymes. The term "purified" as used herein also refers to removal of other components, particularly other proteins and most particularly other enzymes present in the cell of origin of the protease. A protease may be "substantially pure", *i.e.*, substantially free from other components from the organism in which it is produced, *e.g.*, a host organism for recombinantly produced enzyme. Preferably, the protease is at least 75% (w/w) pure, more preferably at least 80%, 85%, 90% or even at least 95% pure. In a still more preferred embodiment, the protease is an at least 98% pure enzyme protein preparation.

However, for the uses according to the invention, the protease need not be that pure. It may *e.g.* include other enzymes, even other proteases, in which case it could be termed a protease preparation.

Enzyme composition

In other aspect, the invention also relates to an enzyme composition comprising papain a second protease, wherein the wherein the second protease is selected from the group consisting of:

- (i) a polypeptide comprising or consisting of amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353;
- (ii) a polypeptide comprising or consisting of an amino acid sequence having at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at

least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, 97%, 98%, 99% or 100% identity to amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353;

- 5 (iii) a variant comprising a substitution, deletion, and/or insertion of one or more (*e.g.*, several) amino acids of amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353; and
- (iv) a fragment of the polypeptide of (i), (ii), or (iii) that has protease activity.

10 Use of the enzyme composition according to the present invention may be combined with use of other enzymes, *e.g.*, other proteases. The additional enzyme may be of any origin, including mammalian and plant, and preferably of microbial (bacterial, yeast or fungal) origin.

Yeast

15 Yeast in the context of the present invention may be any kind of yeast. It may belong to the family *Saccharomycetaceae*. In one particular embodiment, the yeast is a *Saccharomyces*, *e.g.*, *Saccharomyces cerevisiae* or *Saccharomyces uvarum*. In another embodiment, the yeast is a *Kluyveromyces*, *e.g.*, *Kluyveromyces fragilis*. In yet another embodiment, the yeast is *Candida*, *e.g.*, *Candida utilis*, also known as Torula yeast.

20 The yeast to be applied may be in any form, such as yeast especially grown on, *e.g.*, molasses, or spent Brewer's yeast, or yeast collected from alcohol fermentation.

The yeast to be applied may be whole yeast cells or yeast cells which are wholly or partly disrupted or degraded.

25 The process of the invention

A yeast extract, which in the context of the present invention is synonymous to yeast hydrolysate or yeast autolysate, is a soluble extract from yeast comprising hydrolysed protein, which is widely used, *e.g.*, as a flavour enhancer.

30 According to the invention, the yeast extract is produced using exogenous protease for protein hydrolysis. Besides from the added protease, the yeast itself contains a variety of degradative enzymes, including lipases, nucleases, mannanases, glucanases and proteases. The optimum temperatures and pH values for these endogenous enzymes vary, and they may be more or less active under the process conditions according to the present invention.

35 Before addition of the protease, the yeast may be in the form of an aqueous suspension. The dry matter content of the yeast suspension may be in the range 5-50%, such as 10-30%. The pH and temperature of the yeast suspension may be adjusted paying due

regard to the characteristics of the protease in question. In one embodiment, the pH of the yeast suspension is adjusted to be more basic, for example, to be in the range of pH 5.5-9, preferably pH 6-8 or more preferably pH 6-7. The adjustment of pH and temperature may take place before, simultaneous with or after the protease is added.

5 The protease should be applied in an effective amount, *i.e.*, in an amount adequate for sufficient protein hydrolysis.

 In one preferred embodiment, the papain is present at an amount of 0.0125-12.5 mg enzyme protein per g yeast dry matter, preferably at an amount of 0.025-5 mg enzyme protein per g yeast dry matter, preferably at an amount of 0.125-2.5 mg enzyme protein per g yeast dry matter, preferably at amount of 0.25-1.5 mg enzyme protein per g yeast dry matter, and most preferably at an amount of 0.5-1 mg per g yeast dry matter.

 In one preferred embodiment, based on the protease activity the papain is present at an amount of 30-30000 U per g yeast dry matter, preferably at an amount of 60-12000 U per g yeast dry matter, preferably at an amount of 300-6000 U per g yeast dry matter, preferably at amount of 600-3600 U per g yeast dry matter, and most preferably at an amount of 1200-2400 U per g yeast dry matter.

 In one preferred embodiment, the second protease is present at an amount of 0.002-2 mg enzyme protein per g yeast dry matter, preferably at an amount of 0.004-0.4 mg enzyme protein per g yeast dry matter, preferably at amount of 0.02-0.2 mg enzyme protein per g yeast dry matter, and most preferably at an amount of 0.04-0.08 mg enzyme protein per g yeast dry matter.

 In one preferred embodiment, based on the protease activity the second protease is present at an amount of 0.001-1 FLPU-A per g yeast dry matter, preferably at an amount of 0.002-0.2 FLPU-A per g yeast dry matter, preferably at an amount of 0.01-0.1 FLPU-A per g yeast dry matter, and most preferably at an amount of 0.02-0.04 FLPU-A per g yeast dry matter.

 Before or after addition of the protease, the yeast cells may be disrupted, *i.e.*, by raising the temperature. Optionally, chemicals may be added, such as salt or organic solvents. This process is often referred to as plasmolysis. Plasmolysis and protein hydrolysis caused by the exogenous protease may take place simultaneously.

 Self-digestion of the yeast cell contents is often referred to as autolysis. Such self-digestion may take place to varying degrees before or simultaneous with the protein hydrolysis caused by the exogenous protease.

 In one preferred embodiment, plasmolysis, protein hydrolysis caused by the exogenous protease and a certain degree of self-digestion take place simultaneously.

 In another embodiment, plasmolysis and self-digestion with the yeast's own enzymes are performed first, and hydrolysis with the added protease is performed in a separate clarification step.

The incubation following addition of protease may take place at any convenient temperature and incubation time necessary for obtaining the desired degree of protein hydrolysis. Protein hydrolysis in the context of this invention may be performed essentially by the added protease or it may be a combination of the action of the added protease and the self-digestive endogenous proteases of the yeast, i.e., the term protein hydrolysis comprises protein hydrolysis performed by the added protease and protein hydrolysis performed by the yeast's own proteases, which is often referred to as autolysis.

In a preferred embodiment, the incubation temperature is in the range of from about 20°C to about 70°C, preferably from about 40°C to about 60°C, and the incubation time is in the range of from about 1 hour to 48 hours, preferably 12 to 30 hours.

Both pH and temperature can optionally be adjusted to be either higher or lower at any point in the course of the incubation.

Incubation with protease is continued until the desired result is achieved, following which it may or may not be stopped by inactivating the enzyme, e.g., by a heat-treatment step. Such heat-treatment may also serve to pasteurize the yeast extract.

After the proteolytic treatment, the yeast extract may be separated from the cell debris, e.g., by centrifugation and decantation of the supernatant. The yeast extract thus obtained may be concentrated by any method known in the art.

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

Materials and Method

Yeast: fresh block yeast of *Saccharomyces cerevisiae* was available from Angle Yeast China;

Papain: derived from Papaya, available from PangBo China;

AP025 was used as a second protease, which was derived from *Thermoascus aurantiacus* and disclosed as amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353.

JTP196 was used as an another second protease, which is the variant of AP025 (A27K/D79L/Y82F/S87G/D104P/A112P/A126V/D142L) and was described as JTP196 in WO2011/072191.

Protease assays

The activity of Papain was 2400000 U/g enzyme protein, which was determined according to the method described in Chinese National Standard GB/T 23527-2009.

The activity of AP025 was 500 FLPU-A/g enzyme protein, which was determined at pH 4.3 and 65°C using fluorescent detection by EnzChek Protease Assay Kit (FLPU-A). The

EnzChek Protease Assay Kits contain casein derivatives that are heavily labeled with the pH-insensitive red fluorescent BODIPY[®] TR-X dye, resulting in almost total quenching of the conjugate's fluorescence. Protease-catalyzed hydrolysis releases highly fluorescent BODIPY TR-X dye-labelled peptides. The accompanying increase in fluorescence, which can be measured with a microplate fluorescent reader, was proportional to protease activity after incubation at 65°C for 10 minutes. The reaction was stopped with cooling down, and adding 0.1 M HCl to lower the pH.

Example 1 Use of Papain and AP025 in producing a yeast extract

The fresh yeast blocks were mixed with de-ionized water to reach a dry matter content of 13% and stirred for 30 minutes at room temperature. The yeast mixture was heated to 55°C and pH was adjusted to 5.5-6.2. Different groups of enzymes were added to the samples after the temperature and pH were adjusted. The amount of enzyme was dosed on mg enzyme protein/g yeast dry matter (YDM). Hydrolysis was performed for 24 hours at 55°C. Then the samples were inactivated at 85°C for 15 min. And the samples were separated by centrifuge for 15 min at 4000 rpm. The amount of extract was weighed after decantation. The dry matter was measured by direct drying method (Chinese National Standard GB 5009.3-2010). Free amino nitrogen FAN was determined using ninhydrin method (Amino acids are decarboxylised with ninhydrin, ammonia is released as well. The reduced ninhydrin reacts with the unreduced ninhydrin and ammonia to a blue-yellow dye and is measured at 570 nm.). Based on the above measurements the following was calculated:

$$\% \text{Extract Yield} = (\text{g extract after centrifugation}) / (\text{g yeast mixture before centrifugation}) * 100$$

Turbidity of the extract was measured as NTU (Nephelometric Turbidity Units) by a HACH 2100AN turbidity-meter.

Taste evaluation: A panel (e.g., at least 3 well-trained persons) was used to evaluate the bitterness, umami and aroma of the prepared yeast extract.

Results are given in the table 1 below (EP=enzyme protein, YDM=yeast dry matter):

Enzyme	#1	#2	#3	#4		#5		#6
	Papain	Papain	Papain	Papain	AP025	Papain	AP025	AP025
Enzyme dosage (mg EP/ g YDM)	0.5	0.75	1	0.5	0.04	0.75	0.04	0.12
Extract Yield (%) 1 st round	46.00%	46.15 %	48.68%	48.92%		50.63%		43.37%
Extract Yield (%) 2 st round	46.66%	46.84 %	48.82%	49.80%		50.73%		-
Turbidity (NTU)	84	87	83	84		82		83
Taste	same taste: little sweet, strong umami, middle bitterness							strong sourness, light umami

AP025 can synergy with papain in yeast extract application and result in the
5 obviously higher extract yield, and meanwhile there is no influence of the turbidity and taste of
yeast extract.

Example 2 Use of Papain and JTP196 in producing a yeast extract

10 All of the procedures and methods are the same as the procedures and methods
used in example 1, whereas JTP196 was used a second protease. Results are given in the
table 2 below (EP=enzyme protein, YDM=yeast dry matter).

Enzyme	#7	#8	
	Papain	Papain	JTP196
Enzyme dosage (mg EP/ g YDM)	0.75	0.5	0.05
%Extract Yield	47.38%	51.38%	
%FAN/DS	3.99%	4.08%	
Turbidity (NTU)	84	83	
Taste	Little sweet, strong umami, middle bitterness	Same with Papain	

JTP196 also can synergy with papain in yeast extract application and result in the obviously higher extract yield, and meanwhile there is no influence of the turbidity and taste of yeast extract.

CLAIMS

1. A method for producing a yeast extract, comprising:

a) adding an enzyme composition to yeast comprising protein; and

5 b) incubating so as to hydrolyze the protein,

wherein the enzyme composition comprises papain and a second protease, and the second protease is selected from the group consisting of:

(i) a polypeptide comprising or consisting of amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353;

10 (ii) a polypeptide comprising or consisting of an amino acid sequence having at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, 97%, 98%, 99% or 100% identity to amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353;

15 (iii) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., several) amino acids of amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353; and

(iv) a fragment of the polypeptide of (i), (ii), or (iii) that has protease activity.

20

2. The method of claim 1, wherein the second protease is derived from genus *Thermoascus*, preferably from *Thermoascus aurantiacus*, and most preferably from *Thermoascus aurantiacus* CGMCC No. 0670.

25 3. The method of any of the preceding claims, wherein the yeast is *Saccharomyces*, *Kluveromyces* or *Candida*, preferably the yeast is *Saccharomyces cerevisiae* or *Saccharomyces uvarum*.

4. The method of any of the preceding claims, wherein the papain is present at an amount of 0.0125-12.5 mg enzyme protein per g yeast dry matter, preferably at an amount of 0.025-5 mg enzyme protein per g yeast dry matter, preferably at an amount of 0.125-2.5 mg enzyme protein per g yeast dry matter, preferably at amount of 0.25-1.5 mg enzyme protein per g yeast dry matter, and most preferably at an amount of 0.5-1 mg per g yeast dry matter.

35 5. The method of any of the preceding claims, wherein based on the protease activity the papain is present at an amount of 30-30000 U per g yeast dry matter, preferably at an amount of 60-12000 U per g yeast dry matter, preferably at an amount of 300-6000 U per g yeast dry

matter, preferably at amount of 600-3600 U per g yeast dry matter, and most preferably at an amount of 1200-2400 U per g yeast dry matter.

6. The method of any of the preceding claims, wherein the second protease is present at an amount of 0.002-2 mg enzyme protein per g yeast dry matter, preferably at an amount of 0.004-0.4 mg enzyme protein per g yeast dry matter, preferably at amount of 0.02-0.2 mg enzyme protein per g yeast dry matter, and most preferably at an amount of 0.04-0.08 mg enzyme protein per g yeast dry matter.

7. The method of any of the preceding claims, wherein based on the protease activity the second protease is present at an amount of 0.001-1 FLPU-A per g yeast dry matter, preferably at an amount of 0.002-0.2 FLPU-A per g yeast dry matter, preferably at an amount of 0.01-0.1 FLPU-A per g yeast dry matter, and most preferably at an amount of 0.02-0.04 FLPU-A per g yeast dry matter.

8. The method of any of the preceding claims, which further comprises suspending the yeast in an aqueous solution.

9. The method of claim 8, which further comprises adjusting pH of the yeast suspension to be more basic.

10. The method of any of the preceding claims, which further comprises performing centrifugation to obtain a precipitate and a supernatant after step b), preferably wherein the hydrolyzed protein is recovered in the supernatant.

11. An enzyme composition comprising papain and a second protease, wherein the second protease is selected from the group consisting of:

(i) a polypeptide comprising or consisting of amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353;

(ii) a polypeptide comprising or consisting of an amino acid sequence having at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, 97%, 98%, 99% or 100% identity to amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353;

(iii) a variant comprising a substitution, deletion, and/or insertion of one or more (*e.g.*, several) amino acids of amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353; and

(iv) a fragment of the polypeptide of (i), (ii), or (iii) that has protease activity.

12. The enzyme composition of claim 11, wherein the papain or the second protease is defined in any of the preceding claims.

5

13. Use of an enzyme composition to produce a yeast extract, wherein the enzyme composition comprising papain and a second protease, and the second protease is selected from the group consisting of:

10 (i) a polypeptide comprising or consisting of amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353;

(ii) a polypeptide comprising or consisting of an amino acid sequence having at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 15 96%, 97%, 98%, 99% or 100% identity to amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353;

(iii) a variant comprising a substitution, deletion, and/or insertion of one or more (*e.g.*, several) amino acids of amino acids 1-177 of SEQ ID NO: 1 herein or amino acids 1-177 of SEQ ID NO: 2 in WO 2003/048353; and

20 (iv) a fragment of the polypeptide of (i), (ii), or (iii) that has protease activity.

14. The use of claim 13, wherein the yeast, the papain or the second protease is defined in any of the preceding claims.

25 15. A yeast extract produced by the method of any of the preceding claims.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2016/112560

A. CLASSIFICATION OF SUBJECT MATTER

C12N 1/06(2006.01)i; C12N 9/52(2006.01)i

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

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)

CNABS,CNTEXT,SIPOABS,DWPI: NOVOZYMES,produc+,prepar+,manufact+,yeast,extract,protein,papain,protease,amino,acids,polypeptide

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN 101558148 A (NOVOZYMES A/S) 14 October 2009 (2009-10-14) description, page 1, line 21 to page 2, line 23	1-15
A	CN 101513246 A (ANQEL YEAST CO., LTD.) 26 August 2009 (2009-08-26) the whole document	1-15
A	JP 2003325130 A (HASEGAWA T CO., LTD.) 18 November 2003 (2003-11-18) the whole document	1-15
A	WO 2003048353 A1 (NOVOZYMES A/S) 12 June 2003 (2003-06-12) the whole document	1-15
A	KR 20010095724 A (CHOI HYUNG TAEK) 07 November 2001 (2001-11-07) the whole document	1-15



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

“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

22 February 2017

Date of mailing of the international search report

15 March 2017

Name and mailing address of the ISA/CN

STATE INTELLECTUAL PROPERTY OFFICE OF THE
P.R.CHINA
6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing
100088
China

Facsimile No. (86-10)62019451

Authorized officer

WANG, Yongxiu

Telephone No. (86-10)62085475

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2016/112560

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 filed or furnished:

a. (means)

☐

on paper

☒

in electronic form

b. (time)

☒

in the international application as filed

☐

together with the international application in electronic form

☐

subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2016/112560

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
CN	101558148	A	14 October 2009	US	9169474	B2	27 October 2015
				AU	2007338014	B2	24 October 2013
				EP	2097510	A1	09 September 2009
				CA	2670731	A1	03 July 2008
				US	2010093025	A1	15 April 2010
				AU	2007338014	A1	03 July 2008
				WO	2008077890	A1	03 July 2008
				JP	5421117	B2	19 February 2014
				KR	20090101175	A	24 September 2009
				JP	2010512771	A	30 April 2010
				KR	101451841	B1	16 October 2014
				EP	2097510	B1	05 October 2016
				CN	101558148	B	21 May 2014
				BR	PI0720527	A2	07 January 2014
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JP	2011512142	A	21 April 2011				
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TR	201006957	T1	21 January 2011				
CN	101513246	B	05 December 2012				
JP	5398745	B2	29 January 2014				
KR	101255421	B1	17 April 2013				
RU	2455833	C2	20 July 2012				
KR	20100106618	A	01 October 2010				
JP	2003325130	A	18 November 2003	None			
WO	2003048353	A1	12 June 2003	None			
KR	20010095724	A	07 November 2001	None			