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(54) **Title:** METHOD FOR PREPARING STEAMED FLOUR-BASED PRODUCTS BY USING A THERMOSTABLE GLUCOAMYLASE

(57) **Abstract:** The invention relates to methods of producing a steamed flour-based product, said methods comprise: providing a dough or a paste comprising flour and a thermostable glucoamylase at least 70% identical to SEQ ID NO:1, SEQ ID 1X10:6, SEQ ID NO:7 or SEQ ID NO:8; and steaming the dough or the paste to produce a streamed flour-based product, as well as compositions comprising said thermostable glucoamylase.

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METHOD FOR PREPARING STEAMED FLOUR-BASED PRODUCTS BY USING A THERMOSTABLE GLUCOAMYLASE

REFERENCE TO SEQUENCE LISTING

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

FIELD OF THE INVENTION

The invention relates to an enzymatic method for preparing steamed flour-based product, in particular to a method for preparing steamed flour-based products such as steamed bread with a thermostable glucoamylase.

BACKGROUND OF THE INVENTION

Steamed flour-based products such as steamed bread are one of the traditional staple foods of Chinese people, especially in the Northern China. In recent years, with the development of industrialization of food industry, the industrialized production of steamed flour-based products increased gradually.

WO2011/039324 disclosed a method for preparing a steamed bread, comprising the step of making a dough used to prepare steamed bread with one or more maltogenic alpha-amylases, one or more raw starch degrading enzymes, and at least one lipolytic enzyme, wherein the enzymatic method retards the staling of steamed bread products.

In daily life people often buy/make many steamed breads at one time and eat them in several times. The prepared steamed breads are stored at room temperature, cold temperature or frozen for a period of time, before eating again it is often necessary to re-steam the steamed bread in order to restore the softness and mouth feel. However, re-steaming negatively impacts the crumb structure, appearance color, chewiness and/or elasticity of the steamed flour-based products.

Thus, although some advancement in extension of shelf-life of steamed bread have been made using enzymatic treatments. However, there is still a need to provide new enzymatic solutions for improved performance in freshkeeping or anti-staling of steamed flour-based products, even if such steamed flour-based products need to be re-steamed.

SUMMARY OF THE INVENTION

The inventors found that thermostable glucoamylases of the present invention showed greatly improved performance in freshkeeping or anti-staling of steamed flour-based products such as steamed bread, which were prepared by step of steaming the dough.

Another improved performance of the thermostable glucoamylases of the present invention was that they surprisingly and obviously improved the quality of the steamed flour-based products after re-steaming, such as improved appearance whiteness, softness, elasticity, crumb structure, and/or cohesiveness of the re-steamed products.

Another improved performance of the thermostable glucoamylases of the present invention was that they increased the sweetness or sweet taste of the product, the nature sweet is a preferred taste, which also allowed a reduction in the amount of added sugar in traditional recipes.

Accordingly, in a first aspect, a method of producing a steamed flour-based product, comprising:

a) providing a dough or a paste comprising flour and a glucoamylase at least 70% identical to SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8; and

b) steaming the dough or the paste to make the steamed flour-based product.

In a preferred embodiment of the present invention, the said glucoamylase is a mature thermostable variant of a parent glucoamylase.

A second aspect of the invention relates to dough premix or paste premix comprising a dough or a paste, and a mature thermostable variant of a parent glucoamylase as defined in the present invention.

In a preferred embodiment of the present invention, compared with the same conditions as other conditions but without adding the enzyme to the dough/the paste, the steamed flour-based products of the invention have reduced hardness and/or improved elasticity, and thus have an improved storage stability.

In a preferred embodiment of the present invention, compared with the same conditions as other conditions but without adding the enzyme to the dough/the paste, the steamed flour-based product of the present invention has an improved sensory evaluation after being stored at room temperature or low temperature, preferably, for a period of time.

In a preferred embodiment of the present invention, compared with the same conditions as other conditions but without adding the enzyme to the dough/the paste, the re-steamed steamed flour-based products have an improved sensory evaluation.

In a preferred embodiment of the present invention, the sensory evaluation is a comprehensive evaluation preferably, the average value, of softness, elasticity, surface whiteness, crumb structure, moisture, cohesiveness, chewiness and/or sweetness the steamed flour-based products.

Preferably, the glucoamylase of the invention is at least 71% identical to SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8, e.g. at least 72%, e.g. at least 73%, e.g. at least 74%, e.g. at least 75%, e.g. at least 76%, e.g. at least 77%, e.g. at least 78%, e.g. at least 79%, e.g., at least 80%, e.g. at least 81%, e.g. at least 82%, e.g. at least 83%, e.g. at least 84%, e.g.,

at least 85%, e.g. at least 86%, e.g. at least 87%, e.g. at least 88%, e.g. at least 89%, e.g., at least 90%, e.g., at least 91%, e.g., at least 92%, e.g., at least 93%, e.g., at least 94%, e.g., at least 95%, e.g. at least 96%, e.g., at least 97%, e.g., at least 98%, e.g., at least 99% identical to SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8, more preferably, the glucoamylase is a mature thermostable variant of a parent glucoamylase.

FIGURES

Figure 1 shows a multiple alignment of the amino acid sequences of the mature proteins of:

- Wildtype AMG from *Penicillium oxalicum* (PoAMG) of SEQ ID NO:1
- PoAMG variant denoted 'AMG NL' of SEQ ID NO:2
- PoAMG variant denoted 'AMG anPAV498' of SEQ ID NO:3
- PoAMG variant denoted 'AMG JPO001' of SEQ ID NO:4
- PoAMG variant denoted 'AMG JPO124' of SEQ ID NO:5
- PoAMG variant denoted 'AMG JPO172' of SEQ ID NO:6
- Wildtype AMG from *Penicillium miczynskii* (PoAMG) of SEQ ID NO:7
- Wildtype AMG from *Penicillium russellii* (PoAMG) of SEQ ID NO:8
- Wildtype AMG from *Penicillium glabrum* (PoAMG) of SEQ ID NO:9

DETAILED DESCRIPTION OF THE INVENTION

Definitions

In accordance with this detailed description, the following definitions apply. Note that the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise.

Throughout this document, values expressed in a range format should be interpreted in a flexible manner to include not only the numerical values explicitly recited as the limits of the range, but also to include all the individual numerical values or sub-ranges encompassed within that range as if each numerical value and sub-range is explicitly recited. For example, a range of "about 0.1 percent to about 5 percent " or "about 0.1 percent to 5 percent " should be interpreted to include not just about 0.1 percent to about 5 percent, but also the individual values (e.g., 1 percent, 2 percent, 3 percent, and 4 percent) and the sub-ranges (e.g., 0.1 percent to 0.5 percent, 1.1 percent to 2.2 percent, 3.3 percent to 4.4 percent) within the indicated range. The statement "about X to Y" has the same meaning as "about X to about Y," unless indicated otherwise. Likewise, the statement "about X, Y, or about Z" has the same meaning as "about X, about Y, or about Z," unless indicated otherwise.

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 to which this invention belongs.

Glucoamylase: The term glucoamylase (1,4-alpha-D-glucan glucohydrolase, EC 3.2.1.3) is defined as an enzyme, which catalyzes the release of D-glucose from the non-reducing ends of starch or related oligo- and polysaccharide molecules. Glucoamylases are also called amyloglucosidases, and Glucan 1,4-alpha-glucosidase (EC 3.2.1.3), more commonly they are referred to as AMGs. The Glucoamylase Unit (AGU) is defined as the amount of enzyme, which hydrolyses 1 micromole maltose per minute under the standard conditions 37°C, pH 4.3, substrate: maltose 23.2 mM, buffer: acetate 0.1 M, reaction time 5 minutes.

Parent or parent glucoamylase: The term "parent" glucoamylase as used herein means a glucoamylase to which modifications are made to produce the variant glucoamylase of the present invention. This term also refers to the polypeptide with which a variant of the invention is compared. The parent may be a naturally occurring (wild type) polypeptide, or it may even be a variant thereof, prepared by any suitable means. For instance, the parent protein may be a variant of a naturally occurring polypeptide which has been modified or altered in the amino acid sequence. Thus, the parent glucoamylase may have one or more (or one or several) amino acid substitutions, deletions and/or insertions. Thus, the parent glucoamylase may be a variant of a parent glucoamylase. A parent may also be an allelic variant which is a polypeptide encoded by any of two or more alternative forms of a gene occupying the same chromosomal locus.

Mature polypeptide: The term "mature polypeptide" is defined herein as a polypeptide having biological activity that is in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. The mature polypeptide sequence lacks a signal sequence, which may be determined using techniques known in the art (*See*, e.g., Zhang and Henzel, 2004, *Protein Science* 13: 2819-2824). The term "mature polypeptide coding sequence" means a polynucleotide that encodes a mature polypeptide.

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

For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 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:

(Identical Residues x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

Variant: The term “variant” means a polypeptide having glucoamylase 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. The variants of the present invention have at least 20%, *e.g.*, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 100% of the glucoamylase activity of polypeptide of SEQ ID NO 1, SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID NO: 8.

Sensory Evaluation: To perform sensory evaluation, a panel (e.g., at least 3 well-trained persons) is used to assess the qualities of the steamed products.

Improved softness of the steamed product: The term "improved softness of the steamed product" is the opposite of "firmness/hardness" and is defined herein as the property of a steamed product that is more easily compressed compared to a control and is evaluated either empirically by well-trained persons with hand or mouth or measured by, e.g., a texture analyzer (e.g. TAXT2 or TA-XT Plus from Stable Micro Systems Ltd, surrey, UK) as known in the art.

Improved elasticity of the steamed product: The term "improved elasticity of the steamed product" is defined herein as the property of a steamed product that is more easily to recover to original status after pressed compared to a control and is evaluated either empirically by well-trained persons with hand or mouth or measured by, e.g., a texture analyzer (e.g. TAXT2 or TA-XT Plus from Stable Micro Systems Ltd, surrey, UK) as known in the art.

Improved appearance whiteness of the steamed product: The term “improved appearance whiteness of the steamed product” is defined herein as the property of a steamed product with whiter appearance in the surface compared to a control and is usually evaluated visually by well-trained persons.

Improved crumb structure of the steamed product: The term "improved crumb structure of the steamed product" is defined herein as the property of a steamed product with finer cells and/or thinner cell walls in the crumb and/or more uniform/homogenous distribution of cells in the crumb compared to a control and is usually evaluated visually by well-trained persons.

Improved moisture of the steamed product: The term “improved moisture of the steamed product” is defined herein as the property of a steamed product with cooling touch feel or cooling mouth feel compared to a control and is usually evaluated empirically by well-trained persons.

Improved cohesiveness of steamed product: The term “improved cohesiveness of the steamed product” is defined herein as the property of a steamed product with less crumb falling compared to a control and is usually evaluated empirically by well-trained persons.

Improved chewiness of the steamed product: The term “improved chewiness of the steamed product” is defined herein as the property of a steamed product with more effort to chew before swallow compared to a control and is usually evaluated empirically by the skilled baker.

Improved sweetness of the steamed product: The term "improved sweetness of the steamed product" is defined herein as the property of a steamed product with sweeter taste compared to a control and is usually evaluated by well-trained persons.

Thermostability improvement: The thermostability improvement (Td) in °C is a measure of how much the variants have improved in thermostability over their parent glucoamylase under the same conditions, determined as exemplified herein.

Nomenclature

For purposes of the present invention, the nomenclature [Y/F] means that the amino acid at this position may be a tyrosine (Try, Y) or a phenylalanine (Phe, F). Likewise, the nomenclature [V/G/A/I] means that the amino acid at this position may be a valine (Val, V), glycine (Gly, G), alanine (Ala, A) or isoleucine (Ile, I), and so forth for other combinations as described herein. The amino acid X is defined such that it may be any of the 20 natural amino acids, unless otherwise stated.

Conventions for Designation of Variants

For purposes of the present invention, the polypeptide disclosed in SEQ ID NO: 1 is used to determine the corresponding amino acid residue in another glucoamylase. Thus, all mentioned positions and specific substitutions refer to the numbering used in SEQ ID NO: 1. However, the skilled person would recognize that the sequence of any other sequence herein disclosed may also be used to determine the corresponding amino acid residue in another glucoamylase polypeptide. The amino acid sequence of another glucoamylase is aligned with the polypeptide disclosed in SEQ ID NO: 1, and based on the alignment, the amino acid position number corresponding the any amino acid residue in the polypeptide disclosed in SEQ ID No: 1 is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 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.

For proteins of known structure, several tools and resources are available for retrieving and generating structural alignments. For example, the SCOP superfamilies of proteins have been structurally aligned, and those alignments are accessible and downloadable. Two or more protein structures can be aligned using a variety of algorithms such as the distance alignment matrix (Holm and Sander, 1998, *Proteins* 33: 88-96) or combinatorial extension (Shindyalov and Bourne, 1998, *Protein Engineering* 11: 739-747), and implementation of these algorithms can additionally be utilized to query structure databases with a structure of interest in order to discover possible structural homologs (*e.g.*, Holm and Park, 2000, *Bioinformatics* 16: 566-567).

In describing the variants of the present invention, the nomenclature described below is adapted for ease of reference. The accepted IUPAC single letter or three letter amino acid abbreviation is employed.

Substitutions. For an amino acid substitution, the following nomenclature is used: Original amino acid, position, substituted amino acid. Accordingly, the substitution of threonine at position 226 with alanine is designated as "Thr226Ala" or "T226A". Multiple mutations are separated by addition marks ("+"), *e.g.*, "Gly205Arg + Ser411Phe" or "G205R + S411F", representing substitutions at positions 205 and 411 of glycine (G) with arginine (R) and serine (S) with phenylalanine (F), respectively.

Deletions. For an amino acid deletion, the following nomenclature is used: Original amino acid, position, *. Accordingly, the deletion of glycine at position 195 is designated as "Gly195*" or "G195*". Multiple deletions are separated by addition marks ("+"), *e.g.*, "Gly195* + Ser411*" or "G195* + S411*".

Insertions. For an amino acid insertion, the following nomenclature is used: Original amino acid, position, original amino acid, inserted amino acid. Accordingly the insertion of lysine after glycine at position 195 is designated "Gly195GlyLys" or "G195GK". An insertion of multiple amino acids is designated [Original amino acid, position, original amino acid, inserted amino acid #1, inserted amino acid #2; etc.]. For example, the insertion of lysine and alanine after glycine at position 195 is indicated as "Gly195GlyLysAla" or "G195GKA".

In such cases the inserted amino acid residue(s) are numbered by the addition of lower case letters to the position number of the amino acid residue preceding the inserted amino acid residue(s). In the above example, the sequence would thus be:

Parent:	Variant:
195	195 195a 195b
G	G - K - A

Multiple alterations. Variants comprising multiple alterations are separated by addition marks ("+"), *e.g.*, "Arg170Tyr+Gly195Glu" or "R170Y+G195E" representing a substitution of arginine and glycine at positions 170 and 195 with tyrosine and glutamic acid, respectively.

Different alterations. Where different alterations can be introduced at a position, the different alterations are separated by a comma, *e.g.*, "Arg170Tyr, Glu" represents a substitution of arginine at position 170 with tyrosine or glutamic acid. Thus, "Tyr167Gly, Ala + Arg170Gly, Ala" designates the following variants:
"Tyr167Gly+Arg170Gly", "Tyr167Gly+Arg170Ala", "Tyr167Ala+Arg170Gly", and "Tyr167Ala+Arg170Ala".

Detailed Description of the Invention

The first aspect relates to a method of producing a steamed flour-based product, comprising:

a) providing a dough or a paste comprising flour and a glucoamylase at least 70% identical to SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8; and

b) steaming the dough or the paste to make the steamed flour-based product.

In a preferred embodiment of the present invention, the said glucoamylase is a mature thermostable variant of a parent glucoamylase.

The second aspect of the invention relates to dough premix or paste premix comprising a dough or a paste, and a mature thermostable variant of a parent glucoamylase as defined in the present invention.

In a preferred embodiment of the present invention, compared with the same conditions as other conditions but without adding the enzyme to the dough/the paste, after 0.1, 0,25, 0.5, 1, 2, 3, 4, 5, 6 or 7 days of storage at room temperature or low temperature of 4℃, or stored up to 1, 2, 3, 4, 5, 6 months of -20℃, the steamed flour-based products of the invention have reduced hardness and/or improved elasticity, and thus have an improved storage stability.

In a preferred embodiment of the present invention, compared with the same conditions as other conditions but without adding the enzyme to the dough/the paste, after 0.1, 0,25, 0.5, 1, 2, 3, 4, 5, 6 or 7 days of storage at room temperature or low temperature of 4℃, or stored up to 1, 2, 3, 4, 5, 6 months of -20℃, the steamed flour-based product of the present invention has an improved sensory evaluation.

In a preferred embodiment of the present invention, compared with the same conditions as other conditions but without adding the enzyme to the dough/the paste, after 0.1, 0,25, 0.5, 1, 2, 3, 4, 5, 6 or 7 days of storage at room temperature or low temperature of 4℃, or stored up to 1, 2, 3, 4, 5, 6 months of -20℃, re-steaming the steamed flour-based products of the present invention, the re-steamed products have an improved sensory evaluation.

In a preferred embodiment of the present invention, the sensory evaluation is a comprehensive evaluation preferably, the average value, of softness, elasticity, appearance whiteness, crumb structure, moisture, cohesiveness, chewiness and/or sweetness the steamed flour-based products.

In another preferred embodiment of the present invention, the steamed flour-based product has at least the same sweetness or sweet taste as a control product made with double the amount of the mature glucoamylase the amino acid sequence of which is shown in SEQ ID NO:10.

The other aspect relates to a method of producing a boiled flour-based product, comprising:

a) providing a dough comprising flour and a glucoamylase at least 70% identical to SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8; and

b) boiling the dough to make the boiled flour-based product.

In a preferred embodiment of the present invention, the said glucoamylase is a mature thermostable variant of a parent glucoamylase.

Steamed flour-based product:

As used herein, "steamed flour-based products" means any flour-based products prepared by steaming a dough or paste Examples of steamed flour-based products include steamed bread, such as Northern China steamed bread and southern China steamed bread, steamed steamed stuffed bun (bao zi), steamed twisted roll (hua juan), steamed roll (juan zi), steamed dumpling, braised noodles (men mian), spring festival cake (Nian Gao), steamed sponge cake (fa gao), or steamed sponge rice cake (mi fa gao). The steamed flour-based products may contain one or more additional ingredients, such, as meat (e.g., pork, beef, chicken or fish), vegetables (e.g., mushrooms, broccoli, and other green vegetables), fruits (e.g., dates and jujube), candies, cheese, and milk (or other dairy products), and combination thereof.

The present invention relates to a dough or paste comprising a thermostable glucoamylase of the present invention. As used herein "dough" means any dough used to prepare a steamed flour-based product such as a steamed bread. The dough used to prepare a steamed flour-based product may be made from any suitable flour source, e.g., flour sourced from grains, such as, wheat flour, corn flour, rye flour, barley flour, oat flour, rice flour, or sorghum flour, potato flour and combinations thereof (e.g., wheat flour combined with one of the other flour sources; rice flour combined with one of the other flour sources).

The dough of the present invention is usually a fermented dough or a dough to be fermented. The dough can be fermented in various ways, such as by adding dough ingredients such as chemical leavening agent (e.g. sodium bicarbonate) or by adding leavening agent (fermented dough), but it is preferable to ferment the dough by adding a suitable yeast culture, for example, a culture of *Saccharomyces cerevisiae*.

The present invention relates to a flour premix comprising a thermostable glucoamylase of the present invention, such flour premix may comprise any suitable flour source, e.g., flour sourced from grains, such as, wheat flour, corn flour, rye flour, barley flour, oat flour, rice flour, or sorghum flour, potato flour and combinations thereof (e.g., wheat flour combined with one of the other flour sources; rice flour combined with one of the other flour sources).

Methods of preparing steamed bread are well known in the art and include, for example, the "straight dough process" and the "sponge and dough process," non-limiting examples of which are provided under the "Materials and Methods" section below. The process of preparing steamed bread generally involves the sequential steps of dough making (with an optional proofing step),

sheeting, shaping, proofing, and then steaming the dough, which steps are well known in the art. If the optional proofing step is used, preferably more flour is added, and alkali may be added to neutralize acid produced or to be produced during the second proofing step.

Methods of preparing steamed sponge cake are well known in the art and include, for example, a method of preparing steamed sponge cake comprising preparing paste from materials containing flour, fermenting, and steaming to make the steamed sponge cake. Preferably, the paste is a rice paste. Preferably, the flour can be from grains, such as, wheat flour, corn flour, rye flour, barley flour, oat flour, rice flour, or sorghum flour, potato flour and combinations thereof.

Preferably, the finished cake has a delicate network structure, soft taste, pleasant wine aroma and lactic acid flavor produced by fermentation. Preferably, the paste contains a thermostable glucoamylase of the present invention. Preferably, the steamed sponge cake is made from rice, for example, the method comprising: soak the rice in water, and the soaked rice is ground and sieved to obtain rice paste, or a rice paste is made by adding water to steamed sponge cake premix powder, and the rice paste is fermented at 30-40℃ for around 1-25 hours, then sucrose and baking powder may be added, the mixture is evenly mixed, and steam for about 15 minutes to make the steamed sponge cake. The paste is generally fermented by the addition of a suitable yeast culture, for example, a culture of *Saccharomyces cerevisiae* (baker's yeast) or a chemical leavening agent, as are well-known in the art.

In addition to preparing fresh steamed bread dough or steamed bread products, the present invention is directed to method for preparing a frozen steamed bread dough. A frozen steamed bread dough may be advantageous for storage and/or distribution. An example of a method for preparing a frozen steamed bread dough includes the steps of making a dough (with an optional proofing), sheeting, shaping, proofing, and freezing the dough. The present invention is also directed to a frozen steamed bread dough comprising the thermostable glucoamylase of the present invention.

The present invention is particularly useful for preparing steamed bread dough and steamed bread products in industrialized processes, that is, in which the dough used to prepare steamed bread and/or steamed bread products are prepared mechanically using automated or semi-automated equipment. The present invention provides significant advantages in that steamed bread can now be prepared using automated or semi-automated processes in which the steamed bread is stored for distribution and consumer use more than 24 hours after preparation yet substantially maintains the qualities of steamed bread prepared freshly on the same day.

The process of preparing steamed bread generally involves the sequential steps of dough making (and an optional proofing step), sheeting, shaping, proofing, steaming and packaging. If the optional proofing step is used, preferably more flour is added, and alkali may be added to neutralize acid produced or to be produced during the second proofing step. In an industrial steamed bread production process according to the present invention, one or more of

these steps, such as), sheeting, shaping, proofing, steaming and/or packaging, is/are performed using automated or semi-automated equipment.

In one embodiment, the glucoamylase according to the invention may be added to flour or dough or paste in an amount 0.01-1,000 mg enzyme protein (mg EP) per kg flour, preferably in an amount of 0.01-500 mg enzyme protein (mg EP) per kg flour, even more preferably in an amount of 0.1-100 mg enzyme protein (mg EP) per kg flour.

Boiled flour-based product:

As used herein, "Boiled flour-based products" means any flour-based products prepared by boiling dough. Examples of boiled flour-based products include traditional Chinese staple foods such as noodles, dumplings and wonton, rice dumplings and rice noodles, as well as dried products such as dried noodles and semi dry noodles, hot dry noodles, dry rice noodles and semi dry rice noodles as well as wet rice noodles. Flour may be made from any suitable flour source, e.g., flour sourced from grains, such as, rice flour, wheat flour, buckwheat flour, purple rice flour, corn flour, rye flour, barley flour, oat flour, or sorghum flour, potato flour and combinations thereof (e.g., wheat flour combined with one of the other flour sources; rice flour combined with one of the other flour sources). It can be a product with or without stuffing. The stuffing can be meat, vegetables, beans, or their combination. The dough of this kind of products is usually not fermented by yeast. The obtained dough can be processed into products of different shapes through different processes, with or without stuffing.

Boiled flour-based products are usually boiled at an appropriate time according to the thickness of the product or whether there is stuffing and frozen or dry state before eating. Different products are boiled in boiling water for 3-20 minutes to achieve the purpose of fully gelatinization.

In one embodiment, the glucoamylase according to the invention may be added to flour or dough in an amount 0.01-1,000 mg enzyme protein (mg EP) per kg flour, preferably in an amount of 0.01-500 mg enzyme protein (mg EP) per kg flour, even more preferably in an amount of 0.1-100 mg enzyme protein (mg EP) per kg flour.

Glucoamylases

According to the present invention, different types of glucoamylases may be used as parent for the generation of a thermostable glucoamylase variant, e.g, the glucoamylase may be a polypeptide that is encoded by a DNA sequence that is found in a fungal strain of *Aspergillus, Rhizopus*or, *Talaromyces* or *Penicillium*; preferably the DNA sequence that is found in a fungal strain of *Penicillium*, even more preferably the DNA sequence that is found in a fungal strain of *Penicillium oxysporum, Penicillium oxalicum, Penicillium miczynskii, Penicillium russellii or Penicillium glabrum.* Examples of other suitable fungi include *Aspergillus niger, Aspergillus awamori, Aspergillus oryzae, Rhizopus delemar, Rhizopus niveus, Rhizopus oryzae* and

Talaromyces emersonii.

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

In one aspect of the present invention, the glucoamylase, or the parent glucoamylase, may be obtained from *Penicillium* such as, e.g., a *Penicillium oxalicum*, *Penicillum glabrum*, *Penicillium brasilianum*, *Penicillium russellii*, *Penicillium miczynskii*.

In one aspect, the parent fungal glucoamylase, may be a *Penicillium* glucoamylase such as, e.g., a *Penicillium oxalicum* glucoamylase, *Penicillum glabrum* glucoamylase, *Penicillium brasilianum* glucoamylase, *Penicillium russellii* glucoamylase, *Penicillium miczynskii* glucoamylase.

In another aspect, the parent glucoamylase is obtained from *Penicillium oxalicum*, *e.g.*, shown as the glucoamylase of SEQ ID NO: 1. In another aspect, the parent glucoamylase is obtained from *Penicillium oxalicum*, *e.g.*, shown as the glucoamylase of SEQ ID NO: 6. In another aspect, the parent glucoamylase is obtained from *Penicillium oxalicum*, *e.g.*, shown as the glucoamylase of SEQ ID NO: 7. In another aspect, the parent glucoamylase is obtained from *Penicillium oxalicum*, *e.g.*, shown as the glucoamylase of SEQ ID NO: 8.

In one embodiment, the thermostable glucoamylase of the invention is at least 70% identical to SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8, e.g. at least 71%, e.g. at least 72%, e.g. at least 73%, e.g. at least 74%, e.g. at least 75%, e.g. at least 76%, e.g. at least 77%, e.g. at least 78%, e.g. at least 79%, e.g., at least 80%, e.g. at least 81%, e.g. at least 82%, e.g. at least 83%, e.g. at least 84%, e.g., at least 85%, e.g. at least 86%, e.g. at least 87%, e.g. at least 88%, e.g. at least 89%, e.g., at least 90%, e.g., at least 91%, e.g., at least 92%, e.g., at least 93%, e.g., at least 94%, e.g., at least 95%, e.g. at least 96%, e.g., at least 97%, e.g., at least 98%, e.g., at least 99% identical to SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8. In one embodiment, the glucoamylase is a glucoamylase variant or a mature thermostable variant of a parent glucoamylase.

In one embodiment, the mature variant comprises at least one amino acid modification in one or more (several) or all of the positions corresponding to positions 1, 2, 4, 6, 7, 11, 31, 34, 65, 79, 103, 132, 327, 445, 447, 481, 566, 568, 594 and 595 in SEQ ID NO:1.

In one embodiment, wherein the at least one amino acid modification comprises a substitution in one or more or all of the positions corresponding to positions 1, 2, 4, 11, 65, 79 and 327 in SEQ ID NO:1, preferably the at least one amino acid modification comprises a substitution in one or more or all of the positions corresponding to R1A, P2N, P4S, P11F, T65A, K79V and Q327F in SEQ ID NO:1.

In one embodiment, wherein the at least one amino acid modification comprises a substitution in one or more or all of the positions corresponding to positions 1, 6, 7, 31, 34, 79, 103, 132, 445, 447, 481, 566, 568, 594 and 595 in SEQ ID NO:1, preferably the at least one amino acid modification comprises a substitution in one or more or all of the positions corresponding to R1A, G6S, G7T, R31F, K34Y, K79V, S103N, A132P, D445N, V447S, S481P, D566T, T568V, Q594R and F595S in SEQ ID NO:1.

In one embodiment, wherein the at least one amino acid modification comprises a substitution in one or more or all of the positions corresponding to positions 1, 6, 7, 31, 34, 50, 79, 103, 132, 445, 447, 481, 484, 501, 539, 566, 568, 594 and 595 in SEQ ID NO:1, preferably the at least one amino acid modification comprises a substitution in one or more or all of the positions corresponding to R1A, G6S, G7T, R31F, K34Y, E50R, K79V, S103N, A132P, D445N, V447S, S481P, T484P, E501A, N539P, D566T, T568V, Q594R and F595S in SEQ ID NO:1.

In one embodiment, wherein the mature thermostable variant has a thermostability improvement (Td) over its parent of at least 3°C, preferably at least 4°C, 5°C, 6°C, 7°C or 8°C.

In one embodiment, wherein the mature thermostable variant has a relative activity at 91OC of at least 150, preferably at least 200, more preferably at least 250, most preferably at least 300 compared to its parent.

In one embodiment, the thermostable glucoamylase is a glucoamylase variant. In one embodiment, the glucoamylase variant of the present invention has a sequence identity to the polypeptide of SEQ ID NO: 1 or SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID NO: 8 of at least 60% *e.g.,* at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, or 100%, which have glucoamylase activity.

In one embodiment, the amino acid sequence of the glucoamylase variant or the glucoamylase variant of the present invention differs by no more than ten amino acids, *e.g.*, by nine amino acids, by eight amino acids, by seven amino acids, by six amino acids, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from SEQ ID NO: 1, SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID NO: 8 or the mature polypeptide of SEQ ID NO: 1, SEQ ID NO: 6, SEQ IDNO: 7 or SEQ ID NO: 8. In one embodiment. In one embodiment, the mature polypeptide of SEQ ID NO: 1, SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID NO: 8 is shown as SEQ ID NO: 1, SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID NO: 8.

In one embodiment, the amino acid sequence of the glucoamylase, the glucoamylase variant or the glucoamylase variant of the present invention comprises or consists of the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID: 8.

The amino acid changes of variant may be of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the

protein; small deletions, typically of 1-30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

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

Alternatively, the amino acid changes are of such a nature that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may improve the thermal stability of the polypeptide, alter the substrate specificity, change the pH optimum, and the like.

Essential amino acids in a polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for glucoamylase activity to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309: 59-64. The identity of essential amino acids can also be inferred from an alignment with a related polypeptide.

Below is shown the %-identity between the AMG amino acid sequences aligned in Figure 1, and also provided in the sequence list:

P_oxalicum	100.00	99.83	98.99	98.82	96.64	95.97	77.07	77.12
74.32								
AMG_NL	99.83	100.00	99.16	98.99	96.81	96.13	77.07	77.12
74.32								
AMG_anPAV498	98.99	99.16	100.00	99.83	97.65	96.97	76.73	76.95
73.82								

AMG_JPO001	98.82	98.99	99.83	100.00	97.82	97.14	76.73	76.95
73.82								
AMG_JPO124	96.64	96.81	97.65	97.82	100.00	99.33	77.07	77.12
74.32								
AMG_JPO172	95.97	96.13	96.97	97.14	99.33	100.00	76.73	76.78
73.99								
P_miczynskii	77.07	77.07	76.73	76.73	77.07	76.73	100.00	94.75
80.51								
P_russellii	77.12	77.12	76.95	76.95	77.12	76.78	94.75	100.00
79.66								
P_glabrum	74.32	74.32	73.82	73.82	74.32	73.99	80.51	79.66
100.00								

Thermostable variants of the PoAMG have been generated (see table 2 below). In a preferred embodiment, the mature thermostable glucoamylase variant of the invention comprises one or more or all of the combinations of amino acid substitutions listed in table 2 below.

The thermostability improvements (Td) of the variants in table 2 are listed in Table 3, where the Td of the PoAMG variant denoted “anPAV498” (the parent) was set to zero. In a preferred embodiment, the the mature thermostable variant of the invention has a thermostability improvement (Td) over its parent of at least 3°C, preferably at least 4°C, 5°C, 6°C, 7°C or 8°C, preferably determined as exemplified herein.

In another preferred embodiment, the mature thermostable variant of the invention has a relative activity at 91°C of at least 150, preferably at least 200, more preferably at least 250, most preferably at least 300 compared to its parent.

Additional enzymes

Optionally, one or more additional enzymes, such as alpha-amylase, maltogenic amylase, beta amylase, aminopeptidase, carboxypeptidase, catalase, cellulytic enzyme, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, glucan 1,4-alpha-maltotetrahydrolase, glucanase, galactanase, alpha-galactosidase, beta-galactosidase, glucose oxidase, alpha-glucosidase, beta-glucosidase, haloperoxidase, hemicellulytic enzyme, invertase, laccase, lipase, mannanase, mannosidase, oxidase, pectinolytic enzymes, peptidoglutaminase, peroxidase, phospholipase, phytase, polyphenoloxidase, proteolytic

enzyme, ribonuclease, transglutaminase, and xylanase may be used together with the glucoamylase of the present invention.

The additional enzyme(s) may be of any origin, including mammalian, plant, and microbial (bacterial, yeast or fungal) origin.

Enzyme compositions

The mature thermostable glucoamylase variant of the invention as well as any additional enzyme(s) may be added to flour or dough in any suitable form, such as, e.g., in the form of a liquid, in particular a stabilized liquid, or it may be added to flour or dough as a substantially dry powder or granulate.

Granulates may be produced, e.g., as disclosed in US Patent No. 4,106,991 and US Patent No. 4,661,452. Liquid enzyme preparations may, for instance, be stabilized by adding a sugar or sugar alcohol or lactic acid according to established procedures. Other enzyme stabilizers are well-known in the art.

The enzyme(s) may be added to the dough ingredients in any suitable manner, such as individual components (separate or sequential addition of the enzymes) or addition of the enzymes together in one step or one composition.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention as well as combinations of one or more of the embodiments.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties. The present invention is further described by the following example which should not be construed as limiting the scope of the invention.

EXAMPLES

EXAMPLE 1: Construction of PoAMG libraries

PoAMG libraries were constructed as follows:

A forward or reverse primer having NNK or desired mutation(s) at target site(s) with 15 bp overlaps each other were designed. Inverse PCR, which means amplification of entire plasmid DNA sequences by inversely directed primers, were carried out with appropriate template plasmid DNA (e.g. plasmid DNA containing JPO-0001 gene) by the following conditions. The resultant PCR fragments were purified by QIAquick Gel extraction kit [QIAGEN], and then introduced into *Escherichia coli* ECOS Competent E.coli DH5α [NIPPON GENE CO., LTD.]. The plasmid DNAs were extracted from *E. coli* transformants by MagExtractor plasmid extraction kit [TOYOBO], and

then introduced into *A. niger* competent cells.

PCR reaction mix:

PrimeSTAR Max DNA polymerase [TaKaRa]

Total 25 µl

1,0 µl	Template DNA (1 ng/µl)
9,5 µl	H_2O
12,5 µl	2x PrimeSTAR Max pre-mix
1,0 µl	Forward primer (5 µM)
1,0 µl	Reverse primer (5 µM)

PCR program:

98°C/ 2 min

25x (98°C/ 10 sec, 60°C/ 15 sec, 72°C/ 2 min)

10°C/ hold

EXAMPLE 2: Screening for better thermostability

B. subtilis libraries constructed as in EXAMPLE 1 were fermented in either 96-well or 24-well MTP containing COVE liquid medium (2.0 g/L sucrose, 2.0 g/L iso-maltose, 2.0 g/L maltose, 4.9 mg/L, 0.2ml/L 5N NaOH, 10ml/L COVE salt, 10ml/L 1M acetamide), 32°C for 3days. Then, AMG activities in culture supernatants were measured at several temperatures by pNPG assay described as follows.

pNPG thermostability assay:

The culture supernatants containing desired enzymes was mixed with same volume of pH 5.0 200 mM NaOAc buffer. Twenty microliter of this mixture was dispensed into either 96-well plate or 8-strip PCR tube, and then heated by thermal cycler at various temperatures for 30 min. Those samples were mixed with 10 µl of substrate solution containing 0.1% (w/v) pNPG [wako] in pH 5.0 200 mM NaOAc buffer and incubated at 70°C for 20 min for enzymatic reaction. After the reaction, 60 µl of 0.1M Borax buffer was added to stop the reaction. Eighty microliter of reaction supernatant was taken out and its OD_{405} value was read by photometer to evaluate the enzyme activity.

Table 1a. Lists of the relative activity of PoAMG variants when compared with their parent anPAV498 or JPO-0001 (anPAV498 w. leader-/propeptide)

Name	Relative activity of 80°C / 75°C (%)
anPAV498	17%

JPO-004	32%
JPO-005	15%
JPO-006	16%
JPO-007	3%

Name	Relative activity of 80°C / 75°C (%)
AnPav498	13%
JPO-009	16%
JPO-011	15%
JPO-012	15%
JPO-013	17%
JPO-020	20%

Name	Relative activity of 80°C / 70°C (%)
JPO-001	10%
JPO-004	29%
JPO-009	13%
JPO-014	21%
JPO-020	16%
JPO-021	30%
JPO-052	33%

Name	Relative activity of 79°C / 70°C (%)
JPO-001	23%
JPO-021	46%
JPO-022	39%
JPO-023	44%
JPO-025	51%
JPO-027	49%
JPO-029	37%

Name	Relative activity of 77°C / 70°C (%)
JPO-001	72%
JPO-029	82%
JPO-047	80%
JPO-048	90%

JPO-049	84%
JPO-050	86%
JPO-064	87%

name	Relative activity of 79°C / 77°C (%)
JPO-001	36%
JPO-029	51%
JPO-047	45%
JPO-048	81%
JPO-049	53%
JPO-050	58%
JPO-064	65%

Name	Relative activity of 79°C / 77°C (%)
JPO-001	41%
JPO-021	60%
JPO-022	48%
JPO-023	57%
JPO-025	56%
JPO-027	64%
JPO-029	66%
JPO-047	50%
JPO-048	72%
JPO-051	82%
JPO-058	73%
JPO-062	72%
JPO-063	85%
JPO-064	83%

Table 1b. Lists of the relative activity of PoAMG variants when compared with their parent JPO-022

Name	Relative activity of 77°C / 70°C (%)
JPO-022	60%
JPO-027	67%
JPO-042	8%
JPO-044	86%

JPO-045	67%
JPO-046	48%

Name	Relative activity of 77°C / 70°C (%)
JPO-022	76%
JPO-023	75%
JPO-025	80%
JPO-027	84%
JPO-058	92%
JPO-059	88%
JPO-060	86%
JPO-061	83%
JPO-062	87%

Name	Relative activity of 79°C / 77°C (%)
JPO-022	49%
JPO-023	51%
JPO-025	52%
JPO-027	58%
JPO-058	69%
JPO-059	36%
JPO-060	41%
JPO-061	44%
JPO-062	57%

Table 1c. List of the relative activity of PoAMG variants when compared with their parent JPO-063

Name	Relative activity of 79°C / 77°C (%)
JPO-063	91%
JPO-066	96%
JPO-071	89%
JPO-072	84%
JPO-074	103%
JPO-075	86%
JPO-076	92%
JPO-077	95%

JPO-078	88%
JPO-079	100%

Name	Relative activity of 84°C / 80°C (%)
JPO-063	16%
JPO-065	26%
JPO-067	21%
JPO-070	12%
JPO-071	13%
JPO-074	32%
JPO-081	17%
JPO-082	24%
JPO-083	46%
JPO-084	26%
JPO-044	37%

Name	Relative activity of 82°C / 70°C (%)
JPO-063	21%
JPO-093	43%
JPO-081	25%
JPO-088	39%
JPO-094	38%
JPO-096	38%
JPO-106	53%

Name	Relative activity of 83°C / 80°C (%)
JPO-063	46%
JPO-051	44%
JPO-096	64%
JPO-106	88%
JPO-110	81%
JPO-111	100%
JPO-112	86%
JPO-113	83%
JPO-114	47%
JPO-115	90%

Table 1d. List of the relative activity of PoAMG variants when compared with their parent JPO-096

Name	Relative activity of 83°C / 70°C (%)
JPO-082	53%
JPO-088	70%
JPO-091	69%
JPO-092	65%
JPO-093	62%
JPO-094	74%
JPO-095	69%
JPO-096	67%
JPO-097	65%
JPO-098	65%

Name	Relative activity of 83°C / 80°C (%)
JPO-051	20%
JPO-096	43%
JPO-109	51%
JPO-126	33%
JPO-129	48%
JPO-130	18%
JPO-131	51%
JPO-132	34%

Table 1e. List of the relative activity of PoAMG variants when compared with their parents JPO-129

Name	Relative activity of 84°C / 80°C (%)
JPO-129	62%
JPO-156	51%
JPO-160	34%
JPO-161	41%
JPO-162	49%
JPO-163	21%
JPO-164	57%
JPO-165	77%

Table 1f. List of the relative activity of PoAMG variants when compared with their parent JPO-166

Name	Relative activity of 84°C / 75°C (%)
JPO-166	19%
JPO-167	66%
JPO-168	58%
JPO-169	53%
JPO-171	47%
JPO-172	98%

Table 2. Amino acid substitutions in the variants of the PoAMG mature sequence

Name	**Amino acid substitutions**
PoAMG	The wildtype mature AMG from *Penicillium* (SEQ ID NO:1)
AMG NL	K79V
anPAV498	P2N P4S P11F T65A K79V Q327F
JPO-001	R1A P2N P4S P11F T65A K79V Q327F
JPO-018	D75N R77D A78Q
JPO-019	D75S R77G A78W V79D F80Y
JPO-023	R1A K34Y S103N
JPO-024	R1A K34Y D445N V447S
JPO-025	R1A K34Y Y504T
JPO-026	R1A S103N D445N V447S
JPO-027	R1A S103N Y504T
JPO-028	R1A D445N V447S Y504T
JPO-029	R1A K34Y S103N D445N V447S
JPO-044	R1A K34Y S103N D445N V447S E501V Y504T
JPO-047	R1A K34Y S103N Y504T
JPO-048	R1A K34Y S103N D445N V447S D566T
JPO-049	R1A K34Y S103N Q594R F595S
JPO-050	R1A K34Y S103N Y504T Q594R F595S
JPO-051	R1A K34Y S103N D445N V447S Y504T Q594R F595S
JPO-052	R1A S105L
JPO-053	R1A S105E
JPO-055	R1A A132R
JPO-058	R1A K34Y S105L Y504T Q594R F595S

JPO-059	R1A K34Y S103N S105L Y504T Q594R F595S
JPO-060	R1A K34Y S103N S105L Y504T Q594R F595S
JPO-061	R1A K34Y S103N S105L Y504T D566T Q594R F595S
JPO-062	R1A K34Y S103N S105L D445N V447S Y504T D566T Q594R F595S
JPO-063	R1A K34Y S103N S105L A132R D445N V447S Y504T D566T Q594R F595S
JPO-064	R1A K34Y S103N S105L D445N V447S D566T Q594R F595S
JPO-065	R1A K34Y S103N S105L A132R D445N V447S E501V Y504T D566T Q594R R1A F595S
JPO-066	R1A K34Y S103N A132R D445N V447S Y504T D566T Q594R F595S
JPO-069	R1A K34Y S103N S105L A132R D445N V447S Y504T D566T V592T
JPO-071	R1A G6S G7T K34Y S103N S105L A132R D445N V447S Y504T D566T Q594R R1A F595S
JPO-074	R1A K34Y S103N P107L A132R D445N V447S Y504T D566T Q594R F595S
JPO-083	R1A G6S G7T K34Y S103N P107L A132R D445N V447S Y504T D566T Q594R R1A F595S
JPO-084	R1A G6S G7T K34Y S103N P107L A132R D445N V447S Y504T D566T V592T R1A Q594R F595S
JPO-091	R1A G6S R7T K34Y S103N P107L A132P D445N V447S Y504T D566T Q594R F595S
JPO-092	R1A G6S G7T K34Y S103N P107L A132R D445N V447S Y504T D566T T568V Q594R F595S
JPO-093	R1A G6S G7T K34Y S103N P107L A132P D445N V447S Y504T D566T T568V Q594R F595S
JPO-094	R1A G6S G7T K34Y S103N P107L A132R D445N V447S S481P Y504T D566T Q594R F595S
JPO-095	R1A G6S G7T K34Y S103N P107L A132R D445N V447S S481P Y504T D566T T568V Q594R F595S
JPO-096	R1A G6S G7T K34Y S103N P107L A132P D445N V447S D566T T568V Q594R F595S
JPO-097	R1A G6S G7T K34Y S103N P107L T110W A132P D445N V447S Y504T D566T T568V Q594R F595S
JPO-098	R1A G6S G7T K34Y E50R S103N P107L A132P D445N V447S Y504T D566T T568V Q594R F595S
JPO-105	R1A G6S G7T K34Y S103N P107L A132P D445N V447S E501V Y504T
JPO-106	R1A G6S G7T R31F K34Y S103N P107L A132P D445N V447S Y504T D566T T568V Q594R F595S

JPO-108	R1A G6S G7T R31F K34Y S103N P107L A132P D445N V447S S481P Y504T D566T T568V Q594R F595S
JPO-109	R1A G6S G7T K34Y E50R S103N P107L A132P D445N V447S S481P Y504T D566T T568V Q594R F595S
JPO-111	R1A G6S G7T R31F K34Y S103N P107L A132P D445N V447S S481P E501V Y504T D566T T568V Q594R F595S
JPO-112	R1A G6S G7T R31F K34Y S103N P107L A132P D445N V447S S481P D566T T568V Q594R F595S
JPO-114	R1A K34Y D75N R77D A78Q S103N R138L D445N V447S Y504T Q594R F595S
JPO-115	R1A G6S G7T R31F K34Y D75N R77D A78Q S103N P107L A132P D445N V447S S481P Y504T D566T T568V Q594R F595S
JPO-124	R1A G6S G7T R31F K34Y S103N A132P D445N V447S S481P D566T T568V Q594R F595S
JPO-125	R1A G6S G7T K34Y E50R S103N A132P D445N V447S S481P D566T T568V Q594R F595S
JPO-126	R1A R31F K34Y D75N R77D A78Q S103N R138L D445N V447S Y504T Q594R F595S
JPO-127	R1A K34Y D75N R77D A78Q S103N R138L D445N V447S Q594R F595S
JPO-128	R1A G6S G7T R31F K34Y S103N A132P D445N V447S
JPO-129	R1A G6S G7T R31F K34Y E50R S103N A132P D445N V447S S481P D566T T568V Q594R F595S
JPO-130	R1A K34Y E50R D75N R77D A78Q S103N R138L D445N V447S Q594R F595S
JPO-131	R1A G6S G7T R31F K34Y E50R D75N R77D A78Q S103N A132P D445N V447S S481P D566T Q594R F595S
JPO-132	R1A R31F K34Y E50R D75N R77D A78Q S103N R138L D445N V447S Q594R F595S
JPO-133	R1A G6S G7T R31F K34Y E50R D75N R77D A78Q S103N A132P R138L D445N V447S S481P D566T Q594R F595S
JPO-138	R1A R135S
JPO-143	R1A G6S G7T R31F K34Y E50R S103N A132P D445N V447S S481P E501L D566T T568V Q594R F595S
JPO-154	R1A G6S G7T R31F K34Y S103N A132P R138G D445N V447S S481P D566T T568V Q594R F595S
JPO-155	R1A G6S G7T R31F K34Y S103N A132P R138L D445N V447S S481P D566T T568V Q594R F595S
JPO-156	R1A G6S G7T R31F K34Y S103N A132P R138P D445N V447S S481P D566T

	T568V Q594R F595S
JPO-167	R1A G6S G7T R31F K34Y E50R S103N A132P S379P D445N V447S S481P E501A D566T T568V Q594R F595S
JPO-168	R1A G6S G7T R31F K34Y E50R S103N A132P D445N V447S S481P T484P E501A D566T T568V Q594R F595S
JPO-169	R1A G6S G7T R31F K34Y E50R S103N A132P D445N V447S S481P E501A N539P D566T T568V Q594R F595S
JPO-171	R1A G6S G7T R31F K34Y E50R S103N A132P S379P D445N V447S S481P T484P E501A D566T T568V Q594R F595S
JPO-172	R1A G6S G7T R31F K34Y E50R S103N A132P D445N V447S S481P T484P E501A N539P D566T T568V Q594R F595S

EXAMPLE 3: Fermentation of the *Aspergillus niger*

Aspergillus niger strains were fermented on a rotary shaking table in 500 ml baffled flasks containing 100ml MU1 with 4ml 50% urea at 220 rpm, 30°C. The culture broth was centrifuged (10,000 x g, 20 min) and the supernatant was carefully decanted from the precipitates.

EXAMPLE 4: Purification of PoAMG (JPO-001) variants

PoAMG variants were purified by cation exchange chromatography. The peak fractions of each were pooled individually and dialyzed against 20 mM sodium acetate buffer pH 5.0, and then the samples were concentrated using a centrifugal filter unit (Vivaspin Turbo 15, Sartorius). Enzyme concentrations were determined by A280 value.

EXAMPLE 5: Thermostability determination (TSA)

Purified enzyme was diluted with 50 mM sodium acetate buffer pH 5.0 to 0.5 mg/ml and mixed with equal volume of SYPRO Orange (Invitrogen) diluted with Milli-Q water. Eighteen ul of mixture solution were transfer to LightCycler 480 Multiwell Plate 384 (Roche Diagnostics) and the plate was sealed.

Equipment parameters of TSA:

Apparatus: LightCycler 480 Real-Time PCR System (Roche Applied Science)
Scan rate: 0.02°C/sec
Scan range: 37 - 96°C
Integration time: 1.0 sec
Excitation wave length 465 nm
Emission wave length 580 nm

The obtained fluorescence signal was normalized into a range of 0 and 1. The Td was defined as the temperature at which the signal intensity was 0.5. The thermostability improvements are listed in Table 3 with Td of the PoAMG variant denoted anPAV498 as 0.

EXAMPLE 6: PoAMG activity assay

Maltodextrin (DE11) assay by GOD-POD method

Substrate solution

30 g maltodextrin (pindex#2 from MATSUTANI chemical industry Co., Ltd.)
100 ml 120 mM sodium acetate buffer, pH 5.0

Glucose CII test kit (Wako Pure Chemical Industries, Ltd.)

Twenty ul of enzyme samples were mixed with 100 ul of substrate solution and incubated at set temperatures for 2 hours. The samples were cooled down on the aluminum block for 3 min then 10 ul of the reaction solution was mixed with 590 ul of 1 M Tris-HCl pH 8.0 to stop reaction. Ten ul of the solution was mixed with 200 ul of the working solution of the test kit then stand at room temperature for 15 min. The absorbance at A505 was read. The activities are listed in Table 3 as relative activity of the PoAMG variant denoted anPAV498.

Table 3

Variant	Td improvement [°C] (pH5.0, anPAV498 as 0)	Activity at 91°C (anPAV498 as 100)
anPAV498	-	100
JPO-001	1.0	94
JPO-004	2.2	-
JPO-009	0.7	-
JPO-013	1.5	-
JPO-014	2.3	-
JPO-020	1.4	74
JPO-021	2.5	113
JPO-052	2.6	85
JPO-053	0.2	71
JPO-055	1.6	85
JPO-023	3.6	-

JPO-024	2.5	-
JPO-025	3.4	-
JPO-027	2.9	-
JPO-029	3.7	191
JPO-048	4.3	163
JPO-051	5.7	222
JPO-058	4.2	157
JPO-062	4.2	159
JPO-063	5.4	107
JPO-064	4.9	178
JPO-065	7.0	127
JPO-066	6.5	178
JPO-069	4.8	95
JPO-071	6.1	128
JPO-074	6.3	108
JPO-081	5.5	213
JPO-082	5.6	215
JPO-089	6.0	171
JPO-090	5.5	155
JPO-018	0.6	84
JPO-019	0.5	86
JPO-044	6.3	225
JPO-083	6.1	103
JPO-084	4.4	66
JPO-099	6.8	156
JPO-091	6.6	130
JPO-092	6.7	113
JPO-093	6.8	132
JPO-094	6.6	126
JPO-095	6.9	-
JPO-096	5.9	-
JPO-097	5.2	-
JPO-098	5.6	-
JPO-112	8.2	-
JPO-114	5.2	218
JPO-115	8.0	-

JPO-108	8.5	-
JPO-109	7.2	-
JPO-111	8.4	-
JPO-124	8.0	385
JPO-125	6.8	324
JPO-126	6.6	268
JPO-127	4.9	246
JPO-129	8.2	399
JPO-130	5.3	278
JPO-131	7.9	367
JPO-132	6.6	336
JPO-138	6.4	125
JPO-133	6.1	143
JPO-143	8.8	280
JPO-154	7.6	252
JPO-155	8.3	282
JPO-156	8.3	290
JPO-145	8.2	-
JPO-147	8.2	-
JPO-150	8.2	-
JPO-152	8.4	-
JPO-153	9.0	399
JPO-161	6.0	200
JPO-165	8.9	403
JPO-166	7.0	237
JPO-167	9.1	387
JPO-168	9.3	332
JPO-169	9.6	269
JPO-171	9.4	255
JPO-172	9.9	432

Example 7: Use of PoAMG in Steamed Bread

Northern China steamed bread was prepared by a Straight dough process with a recipe according to Table 4 and Table 5. All raw materials used herein are food grade, PoAMG variant JPO-172 (75ppm used herein represents 24.45mg EP/1000g flour) and maltogenic alpha-amylase were used, maltogenic alpha-amylase herein is Novamyl Boost (commercial product of

Novozymes). Briefly, flour, yeast and steamed bread improver were weighed and put into a dough jar (vertical mixer, DIOSNA brand), then enzyme and water were added. The mixture was stirred at a low speed for 6 minutes until the dough was formed and the dough surface was smooth, and the dough was sheeted till it reached the ideal degree. The sheeting times is dependent on hand feeling. After sheeting, dough was weighed out around 110 g and molded it into a steamed bread shape. The molded dough was put into proofing machine for around 40 min under 35℃ (room humidity around 80%). After proofing, the dough was put in a steamer (100℃) and was steamed for 20min. Then the steam was turned off, about 5 mins later, the steamed breads were taken out and cooled at room temperature for 2 hours, after that the prepared steamed bread was packaged with a sealed plastic package, for texture and sensory evaluation.

Re-steaming of steamed bread: The prepared steamed bread was stored at room temperature for 24hrs or in refrigerator at 4℃ for 48hrs, and then it was re-steamed in a steamer (100℃) for around 15 mins. Then the steam was turned off, about 5 mins later, the re-steamed steamed bread was taken out for sensory evaluation.

Table 4. Recipe of Northern Steamed Bread

Recipe	Commercial supplier	weigh percentage % (based on flour)
wheat flour	Wudeili, China	100
Yeast (high activity dry yeast)	Angel Yeast	0.8
Steamed bread improver	Angel Yeast (haodadang brand)	0.5
water		50-52
JPO-172	/	See Table 5
Novamyl Boost	Novozymes A/S	

Table 5:

Batch	JPO-172	Novamyl Boost
A	0	0
B	75 ppm	0
C	0	100 ppm

Sample characterization

Method for determining hardness: The steamed bread was divided by using a slicer (the thickness of each steamed bread slice was 1.2 cm), two sliced steamed bread slices were in one group (the

thickness was 2.4 cm), and were determined by a TA.XT Plus texture analyzer. Gram is used as the unit, the higher the hardness value, represents that the quality of the prepared steamed bread is worse.

Method for determining elasticity: The steamed bread was divided by using a slicer (the thickness of each steamed bread slice was 1.2 cm). Two sliced steamed bread slices were in one group (the thickness was 2.4 cm), and were determined by a TA.XT Plus texture analyzer. % was used as the unit. The higher the elasticity value, represents that the quality of the prepared steamed bread is better.

Sensory evaluation:

To perform sensory evaluation, a panel (5 well-trained persons) was used to assess the qualities of the steamed bread/the re-steamed steamed bread. Parameters, such as, softness, elasticity, appearance whiteness, crumb structure, moisture, cohesiveness, chewiness and/or sweetness were scored. The steamed bread prepared in batch A was scored as 5.0 points and used as the baseline. The mean was taken for a comprehensive evaluation, wherein the higher the score of the mean, represented that the quality of the prepared steamed bread was better.

Table 6. Hardness and elasticity of steamed bread after being stored for 24hrs at room temperature

Batch	Hardness/ g	Elasticity /%
A	2655	54.96
B	1512	63.21
C	1691	60.67

As can be seen from table 6, compared with the control (Batch A), when PoAMG (JPO-172) and Novamyl Boost were used during the preparation of the steamed bread, the hardness of the steamed breads were both significantly reduced, while the elasticity of the steamed bread also were both improved. However, in contrast,] PoAMG (JPO-172) showed a better effect than Novamyl Boost.

Each batch of the steamed breads prepared were subjected to sensory evaluation after being stored at room temperature for 24hr hours, and the results are shown in Table 7. Compared with the control (Batch A), the addition of PoAMG (JPO-172) and Novamyl Boost in the dough improved the sensory evaluation of steamed bread, such as the touch softness, touch elasticity, crumb structure, moisture, cohesiveness, and chewiness were also improved. In addition, PoAMG (JPO-172) also improved the sweetness of steamed bread. In contrast, PoAMG (JPO-172) was significantly better than Novamyl Boost in improving the quality of steamed bread.

Table 7. Sensory evaluation of steamed bread (after 24hrs at room temperature)

Batch	Touch soft-ness	Touch elasti-city	Appea-rance whiteness	Crumb struc-ture	Mois-ture	Cohe-siveness	Chew-iness	Sweet-ness	Ave-rage
A	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
B	6.3	6.0	4.8	5.1	5.5	5.7	5.7	5.7	5.6
C	5.8	5.5	4.3	4.9	5.6	5.7	5.7	5.3	5.3

Each batch of steamed bread prepared were stored at room temperature for 24 hours and then were re-steamed, and then let them cool down to room temperature, after that such re-steamed steamed breads were subjected to sensory evaluation and the results was shown in Table 8.

Table 8. Sensory evaluation of steamed bread (re-steaming after storing at room temperature for 24hrs)

	Touch softness	Touch elasticity	Apperance whiteness	Crumb structure	Chewiness	Sweetness	Average
A	5.0	5.0	5.0	5.0	5.0	5.0	5.0
B	5.6	5.6	5.5	5.6	5.0	5.5	5.5
C	4.8	4.8	4.4	4.6	4.8	5.0	4.7

Each batch of steamed bread prepared were stored at 4℃ for 48 hours and then were re-steamed, and then let them cool down to room temperature, after that such re-steamed steamed breads were subjected to sensory evaluation and the results was shown in Table 9.

Table 9: Sensory evaluation of steamed bread (re-steaming after storage at 4℃ for 48 hours)

	Touch softness	Touch elasticity	Appearance whiteness	Crumb structure	Chewiness	Sweetness	Average

A	5.0	5.0	5.0	5.0	5.0	5.0	5.0
B	5.5	5.5	5.5	5.3	5.2	5.4	5.4
C	4.9	5.0	4.4	5.1	4.7	5.0	4.8

As can be seen from Table 8 and Table 9, compared with the control Batch A, the addition of PoAMG (JPO-172) in the dough obviously improved the sensory evaluation of the re-steamed steamed bread, such as the touch softness, touch elasticity, crumb structure, chewiness and sweetness were all improved. PoAMG (JPO-172) also improved the appearance whiteness of the re-steamed steamed bread. However, in contrast, even when Novamyl Boost was added, the sensory evaluation of re-steamed steamed bread was not good, it was not even as good as that of batch A.

Example 8: Use of POAMG in steamed sponge rice cake

Steamed sponge rice cake was prepared with a recipe according to Table 10 and Table 11, briefly, rice paste was prepared by mixing steamed sponge rice cake premix powder and water in a container, batch A was control without enzyme, PoAMG (JPO-172) was added in the rice paste as Batch B. The mixture was sealed and fermented at 35℃ for 16 hours, then sucrose and baking powder were added after fermentation. After that, the fermented mixture was stirred and put in a mold, and then was steamed in a steamer (100℃) for around 15 minutes. Then the steam was turned off, about 5 mins later, the steamed sponge rice cakes were taken out and cooled at room temperature for 3 hours for texture and sensory evaluation.

Table 10. Recipe of steamed sponge rice cake

Recipe	weigh percentage % (based on Premix powder)
steamed sponge rice cake premix powder (commercially available from Angel Yeast)	100
Water	100
Sucrose	17
Baking powder	2

Table 11:

Batch	Content
A	0

B	65 mg EP/1000g Premix powder

Method for determining hardness: cut a flat surface of steamed sponge rice cake along the outer edge of the mold (the height of each sample was 2.5 cm), and the hardness was determined by a TA.XT Plus texture analyzer. Gram is used as the unit, the higher the hardness value, represents that the quality of the prepared product is worse.

Method for determining elasticity: cut a flat surface of steamed sponge rice cake along the outer edge of the mold (the height of each sample was 2.5 cm), and the elasticity were determined by a TA.XT Plus texture analyzer. The higher the elasticity value, represents that the quality of the prepared product is better.

Sensory evaluation:

To perform sensory evaluation, a panel (5 well-trained persons) was used to assess the qualities of the steamed sponge rice cakes. Parameters, such as, mouthfeel softness, mouthfeel moisture, mouthfeel elasticity, crumb structure, and sweetness were scored. The steamed sponge rice cake prepared in batch A was scored as 5.0 points and used as the baseline. The mean was taken for a comprehensive evaluation, wherein the higher the score of the mean, represented that the quality of the prepared steamed sponge cake was better.

The sensory evaluation of the prepared steamed sponge rice cakes is shown in Table 12. When PoAMG (JPO-172) was added in the paste, mouthfeel softness, mouthfeel moisture, mouthfeel elasticity and sweetness of the prepared steamed sponge cake were improved without crumb structure loss.

Table 12: Sensory evaluation of steamed sponge rice cake

Batch	mouthfeel softness	mouthfeel moisture	Mouthfeel elasticity	crumb structure	Sweetness
A	5	5	5	5	5
B	6.4	6.1	5.9	5	5.5

In addition, the results from the texture analysis can be found in Table 13, when PoAMG (JPO-172 was used in the preparation of the steamed sponge rice cake, the prepared steamed sponge rice cake had reduced hardness and improved elasticity.

Table 13. Hardness and elasticity

Batch	Hardness/g	Elasticity/%
A	3435.25	74.18
B	1810.09	77.08

CLAIMS

1. A method of producing a steamed flour-based product, said method comprising:

a) providing a dough or a paste comprising flour and a glucoamylase at least 70% identical to SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8; and

b) steaming the dough to produce a steamed flour-based product.

2. The method according to claim 1, wherein the glucoamylase is a glucoamylase variant or a mature thermostable variant of a parent glucoamylase.

3. The method according to any of claims 1-2, wherein the steamed flour-based product is selected from: steamed bread, steamed stuffed bun (bao zi), steamed twisted roll (hua juan), steamed roll (juan zi), steamed dumpling, braised noodles (men mian), steamed sponge cake (fa gao), steamed sponge rice cake (mi fa gao), spring festival cake (Nian Gao).

4. The method according to any of claims 1-3, wherein the dough or the paste is made from materials containing flour, preferably, said flour is selected from the group consisting of wheat flour, corn flour, rye flour, barley flour, oat flour, rice flour, or sorghum flour, potato flour and combinations thereof.

5. The method according to any of claims 1-4, wherein the parent glucoamylase is obtained from a species of *Penicillium*, preferably from *Penicillium oxicalum*, *Penicillium miczynskii*, *Penicillium russellii* or *Penicillium glabrum*.

6. The method according to any of claims 1-5, wherein the mature variant comprises at least one amino acid modification in one or more (several) or all of the positions corresponding to positions 1, 2, 4, 6, 7, 11, 31, 34, 65, 79, 103, 132, 327, 445, 447, 481, 566, 568, 594 and 595 in SEQ ID NO:1.

7. The method according to any of claims 1-6, wherein the at least one amino acid modification comprises a substitution in one or more or all of the positions corresponding to positions 1, 2, 4, 11, 65, 79 and 327 in SEQ ID NO:1, preferably the at least one amino acid modification comprises a substitution in one or more or all of the positions corresponding to R1A, P2N, P4S, P11F, T65A, K79V and Q327F in SEQ ID NO:1.

8. The method according to any of claims 1-7, wherein the at least one amino acid modification

comprises a substitution in one or more or all of the positions corresponding to positions 1, 6, 7, 31, 34, 79, 103, 132, 445, 447, 481, 566, 568, 594 and 595 in SEQ ID NO:1, preferably the at least one amino acid modification comprises a substitution in one or more or all of the positions corresponding to R1A, G6S, G7T, R31F, K34Y, K79V, S103N, A132P, D445N, V447S, S481P, D566T, T568V, Q594R and F595S in SEQ ID NO:1.

9. The method according to any of claims 1-8, wherein the at least one amino acid modification comprises a substitution in one or more or all of the positions corresponding to positions 1, 6, 7, 31, 34, 50, 79, 103, 132, 445, 447, 481, 484, 501, 539, 566, 568, 594 and 595 in SEQ ID NO:1, preferably the at least one amino acid modification comprises a substitution in one or more or all of the positions corresponding to R1A, G6S, G7T, R31F, K34Y, E50R, K79V, S103N, A132P, D445N, V447S, S481P, T484P, E501A, N539P, D566T, T568V, Q594R and F595S in SEQ ID NO:1.

10. The method according to any of claims 1-9, wherein the mature thermostable variant has a thermostability improvement (Td) over its parent of at least 3°C, preferably at least 4°C, 5°C, 6°C, 7°C or 8°C.

11. The method according to any of claims 1-10, wherein the mature thermostable variant has a relative activity at 91°C of at least 150, preferably at least 200, more preferably at least 250, most preferably at least 300 compared to its parent.

12. The method according to any of claims 1-11, wherein the glucoamylase, preferably, the mature thermostable variant of a parent glucoamylase is at least 71% identical to SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8, e.g. at least 72%, e.g. at least 73%, e.g. at least 74%, e.g. at least 75%, e.g. at least 76%, e.g. at least 77%, e.g. at least 78%, e.g. at least 79%, e.g., at least 80%, e.g. at least 81%, e.g. at least 82%, e.g. at least 83%, e.g. at least 84%, e.g., at least 85%, e.g. at least 86%, e.g. at least 87%, e.g. at least 88%, e.g. at least 89%, e.g., at least 90%, e.g., at least 91%, e.g., at least 92%, e.g., at least 93%, e.g., at least 94%, e.g., at least 95%, e.g. at least 96%, e.g., at least 97%, e.g., at least 98%, e.g., at least 99%, e.g. at least 100% identical to SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8.

13. The method according to any of claims 1-12, wherein, compared with the same conditions as other conditions but without adding the enzyme to the dough/the paste, after 0.1, 0,25, 0.5, 1, 2, 3, 4, 5, 6 or 7 days of storage at room temperature or low temperature of 4°C, the steamed flour-based product has reduced hardness and/or improved elasticity.

14. The method according to any of claims 1-12, wherein, compared with the same conditions

as other conditions but without adding the enzyme to the dough/the paste, after 0.1, 0,25, 0.5, 1, 2, 3, 4, 5, 6 or 7 days of storage at room temperature or low temperature of 4°C, the steamed flour-based product has an improved sensory evaluation value.

15. The method according to any of claims 1-12, wherein, compared with the same conditions as other conditions but without adding the enzyme to the dough/the paste, after 0.1, 0.25, 0.5, 1, 2, 3, 4, 5, 6 or 7 days of storage at room temperature or low temperature of 4°C, re-steaming the steamed flour-based products, the re-steamed steamed flour-based product has an improved sensory evaluation.

16. The method according to any of claims 1-12, wherein, the sensory evaluation is a comprehensive evaluation preferably, the average value, of softness, elasticity, appearance whiteness, crumb structure, moisture, cohesiveness, chewiness and/or sweetness of the steamed flour-based products or the re-steamed steamed products.

17. A flour composition, a dough premix, or a paste premix, comprising flour and a thermostable glucoamylase, preferably a mature thermostable variant of a parent glucoamylase as defined in any of claims 1-12.

18. A dough comprising flour and a thermostable glucoamylase, preferably a mature thermostable variant of a parent glucoamylase as defined in any of claims 1-12.

Figure 1

```
P_oxalicum      RPDPKGGNLTPFIHKEGERSLQGILDNLGGRGKKTPGTAAGLFIASPNTENPNYYYTWTR
AMG_NL          RPDPKGGNLTPFIHKEGERSLQGILDNLGGRGKKTPGTAAGLFIASPNTENPNYYYTWTR
AMG_anPAV498    RNDSKGGNLTFFIHKEGERSLQGILDNLGGRGKKTPGTAAGLFIASPNTENPNYYYTWTR
JPO001          ANDSKGGNLTFFIHKEGERSLQGILDNLGGRGKKTPGTAAGLFIASPNTENPNYYYTWTR
JPO124          ANDSKSTNLTFFIHKEGERSLQGILDNLGGFGKYTPGTAAGLFIASPNTENPNYYYTWTR
JPO172          ANDSKSTNLTFFIHKEGERSLQGILDNLGGFGKYTPGTAAGLFIASPNTRNPNYYYTWTR
P_miczynskii    NPTSKDAKMASYISKEGQRSIVGITENLGGKGSKTPGTAAGLFIASPNMANPNYYYTWTR
P_russellii     ---SKDGNLASYIAKEGQRSIVGITENLGGKGSKTPGTAAGLFIASPNMANPNYYYTWTR
P_glabrum       ---NPFNSLDSFILKEGARSYQGIIDNLGNKGVKAPGTAAGLFVASPNTANPDYFYTWTR
                       .:  :* *** **  ** :***. *  :********:****  **:*:*****

P_oxalicum      DSALTAKCLIDLFEDSRAKFPIDRKYLETGIRDYVSSQAILQSVSNPSGTLKDGSGLGEP
AMG_NL          DSALTAKCLIDLFEDSRAVFPIDRKYLETGIRDYVSSQAILQSVSNPSGTLKDGSGLGEP
AMG_anPAV498    DSALAAKCLIDLFEDSRAVFPIDRKYLETGIRDYVSSQAILQSVSNPSGTLKDGSGLGEP
JPO001          DSALAAKCLIDLFEDSRAVFPIDRKYLETGIRDYVSSQAILQSVSNPSGTLKDGSGLGEP
JPO124          DSALAAKCLIDLFEDSRAVFPIDRKYLETGIRDYVSSQAILQNVSNPSGTLKDGSGLGEP
JPO172          DSALAAKCLIDLFEDSRAVFPIDRKYLETGIRDYVSSQAILQNVSNPSGTLKDGSGLGEP
P_miczynskii    DSALTFKCLIDLFETSDQDY-ISRKELETDIRNYVSSQAVLQNVSNPSGTLKDGSGLGEP
P_russellii     DSALTFKCLIDLFETSDQDY-ISRKELETDIRNYVSSQAVLQNVSNPSGTLKDGSGLGEP
P_glabrum       DSALTFKCLIDLFDGGSTEFGLKNSELETDIRNYVSSQAVLQNVSNPSGTLEDGTGLGEP
                ****: *******: .   : :... ***.**:******:**.********:**:*****


P_oxalicum      KFEIDLNPFSGAWGRPQRDGPALRATAMITYANYLISHGQKSDVSQVMWPIIANDLAYVG
AMG_NL          KFEIDLNPFSGAWGRPQRDGPALRATAMITYANYLISHGQKSDVSQVMWPIIANDLAYVG
AMG_anPAV498    KFEIDLNPFSGAWGRPQRDGPALRATAMITYANYLISHGQKSDVSQVMWPIIANDLAYVG
JPO001          KFEIDLNPFSGAWGRPQRDGPALRATAMITYANYLISHGQKSDVSQVMWPIIANDLAYVG
JPO124          KFEIDLNPFSGPWGRPQRDGPALRATAMITYANYLISHGQKSDVSQVMWPIIANDLAYVG
JPO172          KFEIDLNPFSGPWGRPQRDGPALRATAMITYANYLISHGQKSDVSQVMWPIIANDLAYVG
P_miczynskii    KFEIDLNPFSGSWGRPQRDGPALRATAMITYADWLISHGSKSEAANIMWPIIANDLAYVG
P_russellii     KFEIDLNPFSGSWGRPQRDGPALRATAMITYADWLVSHGQKSEATNIMWPIIANDLAYVG
P_glabrum       KFEVDLNPFTGSWGRPQRDGPALRATALITYTNYLLSQGQKSEAVNIMWPIISNDLAYVG
                ***:*****:*.***************:***:::*:*:*.**:. ::*****:*******

P_oxalicum      QYWNNTGFDLWEEVDGSSFFTIAVQHRALVEGSQLAKKLGKSCDACDSQPPQILCFLQSF
AMG_NL          QYWNNTGFDLWEEVDGSSFFTIAVQHRALVEGSQLAKKLGKSCDACDSQPPQILCFLQSF
AMG_anPAV498    QYWNNTGFDLWEEVDGSSFFTIAVQHRALVEGSQLAKKLGKSCDACDSQPPQILCFLQSF
JPO001          QYWNNTGFDLWEEVDGSSFFTIAVQHRALVEGSQLAKKLGKSCDACDSQPPQILCFLQSF
JPO124          QYWNNTGFDLWEEVDGSSFFTIAVQHRALVEGSQLAKKLGKSCDACDSQPPQILCFLQSF
JPO172          QYWNNTGFDLWEEVDGSSFFTIAVQHRALVEGSQLAKKLGKSCDACDSQPPQILCFLQSF
P_miczynskii    QYWNNTGFDLWEEVDGSSFFTLAVQHRSLVQGASLAKKLGKSCPACVSQAPQILCFLQSF
P_russellii     QYWNKTGFDLWEEVDGSSFYTLAVQHRALVQGASLAKKLGKSCTACVSQAPQILCFLQSF
P_glabrum       QYWNDTGFDLWEETDGSSFFTLAVQHRALVQGATLAQKLGKSCAACSSQAPQILCFLQSF
                ****.********.*****:*:*****:**:*: **:****** ** **.**********

P_oxalicum      WNGKYITSNINTQASRSGIDLDSVLGSIHTFDPEAACDDATFQPCSARALANHKVYVDSF
AMG_NL          WNGKYITSNINTQASRSGIDLDSVLGSIHTFDPEAACDDATFQPCSARALANHKVYVDSF
AMG_anPAV498    WNGKYITSNINTQASRSGIDLDSVLGSIHTFDPEAACDDATFQPCSARALANHKVYVDSF
JPO001          WNGKYITSNINTQASRSGIDLDSVLGSIHTFDPEAACDDATFQPCSARALANHKVYVDSF
JPO124          WNGKYITSNINTQASRSGIDLDSVLGSIHTFDPEAACDDATFQPCSARALANHKVYVDSF
JPO172          WNGKYITSNINTQASRSGIDLDSVLGSIHTFDPEAACDDATFQPCSARALANHKVYVDSF
P_miczynskii    WNGNYITANINLDTSRSGIDLNSILGSIHTFDPEASCDDSTFQPCSARALANHKVYVDAF
P_russellii     WNGNYITANINLDTSRSGIDLNSILGSIHTFDPEASCDDSTFQPCSARALANHKVYVDAF
P_glabrum       WNGKYITANINLDTSRTGIDANTLLGSIHTFDPEAACDDSTFQPCSARALANHKVYVDAF
                ***:***:*** ::**:*** :::***********:***:******************:*
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Figure 1 – continued

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P_oxalicum      RSIYKINAGLAEGSAANVGRYPEDVYQGGNPWYLATLGASELLYDALYQWDRLGKLEVSE
AMG_NL          RSIYKINAGLAEGSAANVGRYPEDVYQGGNPWYLATLGASELLYDALYQWDRLGKLEVSE
AMG_anPAV498    RSIYKINAGLAEGSAANVGRYPEDVYFGGNPWYLATLGASELLYDALYQWDRLGKLEVSE
JPO001          RSIYKINAGLAEGSAANVGRYPEDVYFGGNPWYLATLGASELLYDALYQWDRLGKLEVSE
JPO124          RSIYKINAGLAEGSAANVGRYPEDVYFGGNPWYLATLGASELLYDALYQWDRLGKLEVSE
JPO172          RSIYKINAGLAEGSAANVGRYPEDVYFGGNPWYLATLGASELLYDALYQWDRLGKLEVSE
P_miczynskii    RSIYKVNAGLSNGSAANVGRYPEDVYQGGNPWYLATLASAELLYDALYQWSKIGKLDVTK
P_russellii     RSIYGVNAGLSNGTAANVGRYPEDVYQGGNPWYLATLAAAELLYDALYQWNQIGKLDVTK
P_glabrum       RSIYKINSGIAEGSPANVGRYPEDVYQGGNPWYLTTLASAELLYDALYQWNKIGGLDVTE
                **** :*:*::::*:.********** *******:**.::**********..:* *:*::

P_oxalicum      TSLSFFKDFDATVKIGSYSRNSKTYKKLTQSIKSYADGFIQLVQQYTPSNGSLAEQYDRN
AMG_NL          TSLSFFKDFDATVKIGSYSRNSKTYKKLTQSIKSYADGFIQLVQQYTPSNGSLAEQYDRN
AMG_anPAV498    TSLSFFKDFDATVKIGSYSRNSKTYKKLTQSIKSYADGFIQLVQQYTPSNGSLAEQYDRN
JPO001          TSLSFFKDFDATVKIGSYSRNSKTYKKLTQSIKSYADGFIQLVQQYTPSNGSLAEQYDRN
JPO124          TSLSFFKDFDATVKIGSYSRNSKTYKKLTQSIKSYADGFIQLVQQYTPSNGSLAEQYDRN
JPO172          TSLSFFKDFDATVKIGSYSRNSKTYKKLTQSIKSYADGFIQLVQQYTPSNGSLAEQYDRN
P_miczynskii    TSLAFFKDFDMAVKTGTYSAHSSTYKSLTSAIRTYADDFIGLVQDYTPSNGSLAEQYDRN
P_russellii     TSLAFFKDFDAAVKTGTYSAHSSAYRTLTSAIRTYADDFISLVQHYTPSNGSLAEQYDRD
P_glabrum       TSLAFFKDFHSSVKTGSYSAHSQTYKTLTSAIRTYADGFVGLVQKYTPANGSLAEQYNRN
                ***:*****  :** *:** :*.:*..**.:*.:***.*: *** ***:********:*:

P_oxalicum      TAAPLSANDLTWSFASFLTATQRRDAVVPPSWGAKSANKVPTTCSASPVVGTYKAPTATF
AMG_NL          TAAPLSANDLTWSFASFLTATQRRDAVVPPSWGAKSANKVPTTCSASPVVGTYKAPTATF
AMG_anPAV498    TAAPLSANDLTWSFASFLTATQRRDAVVPPSWGAKSANKVPTTCSASPVVGTYKAPTATF
JPO001          TAAPLSANDLTWSFASFLTATQRRDAVVPPSWGAKSANKVPTTCSASPVVGTYKAPTATF
JPO124          TAAPLSANDLTWSFASFLTATQRRNASVPPSWGAKSANKVPTTCSASPVVGTYKAPTATF
JPO172          TAAPLSANDLTWSFASFLTATQRRNASVPPSWGAKSANKVPTTCSASPVVGTYKAPTATF
P_miczynskii    TGIPLSANDLTWSYASFITAIQRRASVVPASWGEKSANVVPTICSASPVTGTYQAAPSVF
P_russellii     TGIPLSANDLTWSYASFITAIERRASVVPASWGEKSANVVPTTCSASPVTGTYVAATSVF
P_glabrum       TSVPLSANDLTWSFASFLTAIQRRESIVPGSWGEKSANTVPTTCSASPVTGTYKAATSTF
                *. **********:***:** :** : ** *** **** *** ******.*** *.:.*

P_oxalicum      SSKT-KCVPAKDIVPITFYLIENTYYGENVFMSGNITALGNWDAKKGFPLTANLYTQDQN
AMG_NL          SSKT-KCVPAKDIVPITFYLIENTYYGENVFMSGNITALGNWDAKKGFPLTANLYTQDQN
AMG_anPAV498    SSKT-KCVPAKDIVPITFYLIENTYYGENVFMSGNITALGNWDAKKGFPLTANLYTQDQN
JPO001          SSKT-KCVPAKDIVPITFYLIENTYYGENVFMSGNITALGNWDAKKGFPLTANLYTQDQN
JPO124          PSKT-KCVPAKDIVPITFYLIENTYYGENVFMSGNITALGNWDAKKGFPLTANLYTQDQN
JPO172          PSKP-KCVPAKDIVPITFYLIANTYYGENVFMSGNITALGNWDAKKGFPLTANLYTQDQP
P_miczynskii    PTTT-GCVPATSIVPITFYLTETTFYGENVYMTGNISALGNWDTNNGFPLTANLYTDSDH
P_russellii     PTTT-GCVPATSIVPITFYLTESTFYGENVYMTGNISALGNWDTSSGFPLTANLYTDSDH
P_glabrum       PTSTAGCVPATDVVPITFYLIENTYYGENVYMTGNISALGNWDTSDGLALDAGLYTETDN
                .:..  ****..:*******  .*:*****:*:***:******:..*:.* *.***: :

P_oxalicum      LWFASVEFIPAGTPFEYKYYKVEPNGDI-TWEKGPNRVFVAPTGCPVQPHSNDVWQF
AMG_NL          LWFASVEFIPAGTPFEYKYYKVEPNGDI-TWEKGPNRVFVAPTGCPVQPHSNDVWQF
AMG_anPAV498    LWFASVEFIPAGTPFEYKYYKVEPNGDI-TWEKGPNRVFVAPTGCPVQPHSNDVWQF
JPO001          LWFASVEFIPAGTPFEYKYYKVEPNGDI-TWEKGPNRVFVAPTGCPVQPHSNDVWQF
JPO124          LWFASVEFIPAGTPFEYKYYKVEPNGTI-VWEKGPNRVFVAPTGCPVQPHSNDVWRS
JPO172          LWFASVEFIPAGTPFEYKYYKVEPNGTI-VWEKGPNRVFVAPTGCPVQPHSNDVWRS
P_miczynskii    LWFASVELVPAGTPFEYKYYKVEPNGTV-IWENGENRVYVAPTGCPIQPSQTDVWR-
P_russellii     LWFASVELVPAGTPFEYKYYKVEPNGTV-IWENGENRVYVAPTGCPIQPSQTDIWR-
P_glabrum       LWFGTLELVTAGTPFEYKYYKIEPNGTVTIWESGDNRVSVVPTGCPIQPSLHDVWRS
                ***.::*::.**********:**** :  **.* *** *.*****:**   *:*.
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Sequence Listing

1	**Sequence Listing Information**	
1-1	File Name	15399-WO-PCT[2].xml
1-2	DTD Version	V1_3
1-3	Software Name	WIPO Sequence
1-4	Software Version	2.2.0
1-5	Production Date	2022-11-23
1-6	Original free text language code	en
1-7	Non English free text language code	
2	**General Information**	
2-1	Current application: IP Office	
2-2	Current application: Application number	
2-3	Current application: Filing date	
2-4	Current application: Applicant file reference	15399-WO-PCT[2]
2-5	Earliest priority application: IP Office	CN
2-6	Earliest priority application: Application number	PCT/CN2022/088680
2-7	Earliest priority application: Filing date	2022-04-24
2-8en	Applicant name	Novozymes A/S
2-8	Applicant name: Name Latin	
2-9en	Inventor name	
2-9	Inventor name: Name Latin	
2-10en	Invention title	Method for preparing steamed flour-based products by using a thermostable glucoamylase
2-11	Sequence Total Quantity	10

3-1	**Sequences**	
3-1-1	Sequence Number [ID]	1
3-1-2	Molecule Type	AA
3-1-3	Length	595
3-1-4	Features Location/	**source 1..595**
	Qualifiers	mol_type=protein
		organism=Penicillium oxalicum
	NonEnglishQualifier Value	
3-1-5	Residues	RPDPKGGNLT PFIHKEGERS LQGILDNLGG RGKKTPGTAA GLFIASPNTE NPNYYYTWTR 60 DSALTAKCLI DLFEDSRAKF PIDRKYLETG IRDYVSSQAI LQSVSNPSGT LKDGSGLGEP 120 KFEIDLNPFS GAWGRPQRDG PALRATAMIT YANYLISHGQ KSDVSQVMWP IIANDLAYVG 180 QYWNNTGFDL WEEVDGSSFF TIAVQHRALV EGSQLAKKLG KSCDACDSQP PQILCFLQSF 240 WNGKYITSNI NTQASRSGID LDSVLGSIHT FDPEAACDDA TFQPCSARAL ANHKVYVDSF 300 RSIYKINAGL AEGSAANVGR YPEDVYQGGN PWYLATLGAS ELLYDALYQW DRLGKLEVSE 360 TSLSFFKDFD ATVKIGSYSR NSKTYKKLTQ SIKSYADGFI QLVQQYTPSN GSLAEQYDRN 420 TAAPLSANDL TWSFASFLTA TQRRDAVVPP SWGAKSANKV PTTCSASPVV GTYKAPTATF 480 SSKTKCVPAK DIVPITFYLI ENTYYGENVF MSGNITALGN WDAKKGFPLT ANLYTQDQNL 540 WFASVEFIPA GTPFEYKYYK VEPNGDITWE KGPNRVFVAP TGCPVQPHSN DVWQF 595
3-2	**Sequences**	
3-2-1	Sequence Number [ID]	2
3-2-2	Molecule Type	AA
3-2-3	Length	595
3-2-4	Features Location/	**REGION 1..595**
	Qualifiers	note=AMG NL
		source 1..595
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-2-5	Residues	RPDPKGGNLT PFIHKEGERS LQGILDNLGG RGKKTPGTAA GLFIASPNTE NPNYYYTWTR 60 DSALTAKCLI DLFEDSRAVF PIDRKYLETG IRDYVSSQAI LQSVSNPSGT LKDGSGLGEP 120 KFEIDLNPFS GAWGRPQRDG PALRATAMIT YANYLISHGQ KSDVSQVMWP IIANDLAYVG 180 QYWNNTGFDL WEEVDGSSFF TIAVQHRALV EGSQLAKKLG KSCDACDSQP PQILCFLQSF 240 WNGKYITSNI NTQASRSGID LDSVLGSIHT FDPEAACDDA TFQPCSARAL ANHKVYVDSF 300 RSIYKINAGL AEGSAANVGR YPEDVYQGGN PWYLATLGAS ELLYDALYQW DRLGKLEVSE 360 TSLSFFKDFD ATVKIGSYSR NSKTYKKLTQ SIKSYADGFI QLVQQYTPSN GSLAEQYDRN 420 TAAPLSANDL TWSFASFLTA TQRRDAVVPP SWGAKSANKV PTTCSASPVV GTYKAPTATF 480 SSKTKCVPAK DIVPITFYLI ENTYYGENVF MSGNITALGN WDAKKGFPLT ANLYTQDQNL 540 WFASVEFIPA GTPFEYKYYK VEPNGDITWE KGPNRVFVAP TGCPVQPHSN DVWQF 595
3-3	**Sequences**	
3-3-1	Sequence Number [ID]	3
3-3-2	Molecule Type	AA
3-3-3	Length	595
3-3-4	Features Location/	**REGION 1..595**
	Qualifiers	note=AMG anPAV498
		source 1..595
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-3-5	Residues	RNDSKGGNLT FFIHKEGERS LQGILDNLGG RGKKTPGTAA GLFIASPNTE NPNYYYTWTR 60 DSALAAKCLI DLFEDSRAVF PIDRKYLETG IRDYVSSQAI LQSVSNPSGT LKDGSGLGEP 120 KFEIDLNPFS GAWGRPQRDG PALRATAMIT YANYLISHGQ KSDVSQVMWP IIANDLAYVG 180 QYWNNTGFDL WEEVDGSSFF TIAVQHRALV EGSQLAKKLG KSCDACDSQP PQILCFLQSF 240 WNGKYITSNI NTQASRSGID LDSVLGSIHT FDPEAACDDA TFQPCSARAL ANHKVYVDSF 300 RSIYKINAGL AEGSAANVGR YPEDVYFGGN PWYLATLGAS ELLYDALYQW DRLGKLEVSE 360 TSLSFFKDFD ATVKIGSYSR NSKTYKKLTQ SIKSYADGFI QLVQQYTPSN GSLAEQYDRN 420 TAAPLSANDL TWSFASFLTA TQRRDAVVPP SWGAKSANKV PTTCSASPVV GTYKAPTATF 480 SSKTKCVPAK DIVPITFYLI ENTYYGENVF MSGNITALGN WDAKKGFPLT ANLYTQDQNL 540 WFASVEFIPA GTPFEYKYYK VEPNGDITWE KGPNRVFVAP TGCPVQPHSN DVWQF 595
3-4	**Sequences**	
3-4-1	Sequence Number [ID]	4
3-4-2	Molecule Type	AA
3-4-3	Length	595
3-4-4	Features Location/	**REGION 1..595**
	Qualifiers	note=AMG JPO-001
		source 1..595

		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-4-5	Residues	ANDSKGGNLT FFIHKEGERS LQGILDNLGG RGKKTPGTAA GLFIASPNTE NPNYYYTWTR 60 DSALAAKCLI DLFEDSRAVF PIDRKYLETG IRDYVSSQAI LQSVSNPSGT LKDGSGLGEP 120 KFEIDLNPFS GAWGRPQRDG PALRATAMIT YANYLISHGQ KSDVSQVMWP IIANDLAYVG 180 QYWNNTGFDL WEEVDGSSFF TIAVQHRALV EGSQLAKKLG KSCDACDSQP PQILCFLQSF 240 WNGKYITSNI NTQASRSGID LDSVLGSIHT FDPEAACDDA TFQPCSARAL ANHKVYVDSF 300 RSIYKINAGL AEGSAANVGR YPEDVYFGGN PWYLATLGAS ELLYDALYQW DRLGKLEVSE 360 TSLSFFKDFD ATVKIGSYSR NSKTYKKLTQ SIKSYADGFI QLVQQYTPSN GSLAEQYDRN 420 TAAPLSANDL TWSFASFLTA TQRRDAVVPP SWGAKSANKV PTTCSASPVV GTYKAPTATF 480 SSKTKCVPAK DIVPITFYLI ENTYYGENVF MSGNITALGN WDAKKGFPLT ANLYTQDQNL 540 WFASVEFIPA GTPFEYKYYK VEPNGDITWE KGPNRVFVAP TGCPVQPHSN DVWQF 595
3-5	**Sequences**	
3-5-1	Sequence Number [ID]	5
3-5-2	Molecule Type	AA
3-5-3	Length	595
3-5-4	Features Location/	**REGION 1..595**
	Qualifiers	note=AMG JPO-124
		source 1..595
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-5-5	Residues	ANDSKSTNLT FFIHKEGERS LQGILDNLGG FGKYTPGTAA GLFIASPNTE NPNYYYTWTR 60 DSALAAKCLI DLFEDSRAVF PIDRKYLETG IRDYVSSQAI LQNVSNPSGT LKDGSGLGEP 120 KFEIDLNPFS GPWGRPQRDG PALRATAMIT YANYLISHGQ KSDVSQVMWP IIANDLAYVG 180 QYWNNTGFDL WEEVDGSSFF TIAVQHRALV EGSQLAKKLG KSCDACDSQP PQILCFLQSF 240 WNGKYITSNI NTQASRSGID LDSVLGSIHT FDPEAACDDA TFQPCSARAL ANHKVYVDSF 300 RSIYKINAGL AEGSAANVGR YPEDVYFGGN PWYLATLGAS ELLYDALYQW DRLGKLEVSE 360 TSLSFFKDFD ATVKIGSYSR NSKTYKKLTQ SIKSYADGFI QLVQQYTPSN GSLAEQYDRN 420 TAAPLSANDL TWSFASFLTA TQRRNASVPP SWGAKSANKV PTTCSASPVV GTYKAPTATF 480 PSKTKCVPAK DIVPITFYLI ENTYYGENVF MSGNITALGN WDAKKGFPLT ANLYTQDQNL 540 WFASVEFIPA GTPFEYKYYK VEPNGTIVWE KGPNRVFVAP TGCPVQPHSN DVWRS 595
3-6	**Sequences**	
3-6-1	Sequence Number [ID]	6
3-6-2	Molecule Type	AA
3-6-3	Length	595
3-6-4	Features Location/	**REGION 1..595**
	Qualifiers	note=AMG JPO-172
		source 1..595
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-6-5	Residues	ANDSKSTNLT FFIHKEGERS LQGILDNLGG FGKYTPGTAA GLFIASPNTR NPNYYYTWTR 60 DSALAAKCLI DLFEDSRAVF PIDRKYLETG IRDYVSSQAI LQNVSNPSGT LKDGSGLGEP 120 KFEIDLNPFS GPWGRPQRDG PALRATAMIT YANYLISHGQ KSDVSQVMWP IIANDLAYVG 180 QYWNNTGFDL WEEVDGSSFF TIAVQHRALV EGSQLAKKLG KSCDACDSQP PQILCFLQSF 240 WNGKYITSNI NTQASRSGID LDSVLGSIHT FDPEAACDDA TFQPCSARAL ANHKVYVDSF 300 RSIYKINAGL AEGSAANVGR YPEDVYFGGN PWYLATLGAS ELLYDALYQW DRLGKLEVSE 360 TSLSFFKDFD ATVKIGSYSR NSKTYKKLTQ SIKSYADGFI QLVQQYTPSN GSLAEQYDRN 420 TAAPLSANDL TWSFASFLTA TQRRNASVPP SWGAKSANKV PTTCSASPVV GTYKAPTATF 480 PSKPKCVPAK DIVPITFYLI ANTYYGENVF MSGNITALGN WDAKKGFPLT ANLYTQDQPL 540 WFASVEFIPA GTPFEYKYYK VEPNGTIVWE KGPNRVFVAP TGCPVQPHSN DVWRS 595
3-7	**Sequences**	
3-7-1	Sequence Number [ID]	7
3-7-2	Molecule Type	AA
3-7-3	Length	593
3-7-4	Features Location/	**source 1..593**
	Qualifiers	mol_type=protein
		organism=Penicillium miczynskii
	NonEnglishQualifier Value	
3-7-5	Residues	NPTSKDAKMA SYISKEGQRS IVGITENLGG KGSKTPGTAA GLFIASPNMA NPNYYYTWTR 60 DSALTFKCLI DLFETSDQDY ISRKELETDI RNYVSSQAVL QNVSNPSGTL KDGSGLGEPK 120 FEIDLNPFSG SWGRPQRDGP ALRATAMITY ADWLISHGSK SEAANIMWPI IANDLAYVGQ 180 YWNNTGFDLW EEVDGSSFFT LAVQHRSLVQ GASLAKKLGK SCPACVSQAP QILCFLQSFW 240

		NGNYITANIN LDTSRSGIDL NSILGSIHTF DPEASCDDST FQPCSARALA NHKVYVDAFR 300 SIYKVNAGLS NGSAANVGRY PEDVYQGGNP WYLATLASAE LLYDALYQWS KIGKLDVTKT 360 SLAFFKDFDM AVKTGTYSAH SSTYKSLTSA IRTYADDFIG LVQDYTPSNG SLAEQYDRNT 420 GIPLSANDLT WSYASFITAI QRRASVVPAS WGEKSANVVP TICSASPVTG TYQAAPSVFP 480 TTTGCVPATS IVPITFYLTE TTFYGENVYM TGNISALGNW DTNNGFPLTA NLYTDSDHLW 540 FASVELVPAG TPFEYKYYKV EPNGTVIWEN GENRVYVAPT GCPIQPSQTD VWR 593
3-8	**Sequences**	
3-8-1	Sequence Number [ID]	8
3-8-2	Molecule Type	AA
3-8-3	Length	590
3-8-4	Features Location/	**source 1..590**
	Qualifiers	mol_type=protein
		organism=Penicillium russellii
	NonEnglishQualifier Value	
3-8-5	Residues	SKDGNLASYI AKEGQRSIVG ITENLGGKGS KTPGTAAGLF IASPNMANPN YYYTWTRDSA 60 LTFKCLIDLF ETSDQDYISR KELETDIRNY VSSQAVLQNV SNPSGTLKDG SGLGEPKFEI 120 DLNPFSGSWG RPQRDGPALR ATAMITYADW LVSHGQKSEA TNIMWPIIAN DLAYVGQYWN 180 KTGFDLWEEV DGSSFYTLAV QHRALVQGAS LAKKLGKSCT ACVSQAPQIL CFLQSFWNGN 240 YITANINLDT SRSGIDLNSI LGSIHTFDPE ASCDDSTFQP CSARALANHK VYVDAFRSIY 300 GVNAGLSNGT AANVGRYPED VYQGGNPWYL ATLAAAELLY DALYQWNQIG KLDVTKTSLA 360 FFKDFDAAVK TGTYSAHSSA YRTLTSAIRT YADDFISLVQ HYTPSNGSLA EQYDRDTGIP 420 LSANDLTWSY ASFITAIERR ASVVPASWGE KSANVVPTTC SASPVTGTYV AATSVFPTTT 480 GCVPATSIVP ITFYLTESTF YGENVYMTGN ISALGNWDTS SGFPLTANLY TDSDHLWFAS 540 VELVPAGTPF EYKYYKVEPN GTVIWENGEN RVYVAPTGCP IQPSQTDIWR 590
3-9	**Sequences**	
3-9-1	Sequence Number [ID]	9
3-9-2	Molecule Type	AA
3-9-3	Length	594
3-9-4	Features Location/	**source 1..594**
	Qualifiers	mol_type=protein
		organism=Penicillium glabrum
	NonEnglishQualifier Value	
3-9-5	Residues	NPFNSLDSFI LKEGARSYQG IIDNLGNKGV KAPGTAAGLF VASPNTANPD YFYTWTRDSA 60 LTFKCLIDLF DGGSTEFGLK NSELETDIRN YVSSQAVLQN VSNPSGTLED GTGLGEPKFE 120 VDLNPFTGSW GRPQRDGPAL RATALITYTN YLLSQGQKSE AVNIMWPIIS NDLAYVGQYW 180 NDTGFDLWEE TDGSSFFTLA VQHRALVQGA TLAQKLGKSC AACSSQAPQI LCFLQSFWNG 240 KYITANINLD TSRTGIDANT LLGSIHTFDP EAACDDSTFQ PCSARALANH KVYVDAFRSI 300 YKINSGIAEG SPANVGRYPE DVYQGGNPWY LTTLASAELL YDALYQWNKI GGLDVTETSL 360 AFFKDFHSSV KTGSYSAHSQ TYKTLTSAIR TYADGFVGLV QKYTPANGSL AEQYNRNTSV 420 PLSANDLTWS FASFLTAIQR RESIVPGSWG EKSANTVPTT CSASPVTGTY KAATSTFPTS 480 TAGCVPATDV VPITFYLIEN TYYGENVYMT GNISALGNWD TSDGLALDAG LYTETDNLWF 540 GTLELVTAGT PFEYKYYKIE PNGTVTIWES GDNRVSVVPT GCPIQPSLHD VWRS 594
3-10	**Sequences**	
3-10-1	Sequence Number [ID]	10
3-10-2	Molecule Type	AA
3-10-3	Length	591
3-10-4	Features Location/	**source 1..591**
	Qualifiers	mol_type=protein
		organism=Rasamsonia emersonii
	NonEnglishQualifier Value	
3-10-5	Residues	ATGSLDSFLA TETPIALQGV LNNIGPNGAD VAGASAGIVV ASPSRSDPNY FYSWTRDAAL 60 TAKYLVDAFI AGNKDLEQTI QQYISAQAKV QTISNPSGDL STGGLGEPKF NVNETAFTGP 120 WGRPQRDGPA LRATALIAYA NYLIDNGEAS TADEIIWPIV QNDLSYITQY WNSSTFDLWE 180 EVEGSSFFTT AVQHRALVEG NALATRLNHT CSNCVSQAPQ VLCFLQSYWT GSYVLANFGG 240 SGRSGKDVNS ILGSIHTFDP AGGCDDSTFQ PCSARALANH KVVTDSFRSI YAINSGIAEG 300 SAVAVGRYPE DVYQGGNPWY LATAAAAEQL YDAIYQWKKI GSISITDVSL PFFQDIYPSA 360 AVGTYNSGST TFNDIISAVQ TYGDGYLSIV EKYTPSDGSL TEQFSRTDGT PLSASALTWS 420 YASLLTASAR RQSVVPASWG ESSASSVPAV CSATSATGPY STATNTVWPS SGSGSSTTTS 480 SAPCTTPTSV AVTFDEIVST SYGETIYLAG SIPELGNWST ASAIPLRADA YTNSNPLWYV 540 TVNLPPGTSF EYKFFKNQTD GTIVWEDDPN RSYTVPAYCG QTTAILDDSW Q 591