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(54) Title: AMYLASE POLYPEPTIDES

(57) Abstract: This invention relates to polypeptides, more specifically amylase polypeptides and nucleic acids encoding these, and their uses e.g. as non-maltogenic exoamylases in producing food or feed products.



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AMYLASE POLYPEPTIDES

FIELD OF THE INVENTION

This invention relates to polypeptides, specifically amylase polypeptides and nucleic acids encoding these, and their uses e.g. as non-maltogenic exoamylases in producing food or feed products.

BACKGROUND OF THE INVENTION

Improved amylases can ameliorate problems inherent in certain processes, such as in conversion of vegetable starches or baking.

Crystallisation of amylopectin takes place in starch granules days after baking, which leads to increased firmness of bread and causes bread staling. When bread stales, bread loses crumb softness and crumb moisture. As a result, crumbs become less elastic, and bread develops a leathery crust.

Enzymatic hydrolysis (by amylases, for example) of amylopectin side chains can reduce crystallization and increase anti-staling. Crystallization depends upon the length of amylopectin side chains: the longer the side chains, the greater the crystallization. Most starch granules are composed of a mixture of two polymers: amylopectin and amylose, of which about 75% is amylopectin. Amylopectin is a very large, branched molecule consisting of chains of α -D-glucopyranosyl units joined by (1-4) linkages, where the chains are attached by α -D-(1-6) linkages to form branches. Amylose is a linear chain of (1-4) linked α -D-glucopyranosyl units having few α -D-(1-6) branches.

Baking of farinaceous bread products such as white bread, bread made from bolted rye flour and wheat flour and rolls is accomplished by baking the bread dough at oven temperatures in the range of from 180 to 250°C for about 15 to 60 minutes. During the baking process a steep temperature gradient (200 to 120°C) prevails over the outer dough layers where the crust of the baked product is developed. However, due to steam, the temperature in the crumb is only about 100°C at the end of the baking process. Above temperatures of about 85°C, enzyme inactivation can take place and the enzyme will have no anti-staling properties. Only thermostable amylases, thus, are able to modify starch efficiently during baking.

Endoamylase activity can negatively affect the quality of the final bread product by producing a sticky or gummy crumb due to the accumulation of branched dextrins. Exo-amylase activity is preferred, because it accomplishes the desired modification of starch that leads to retardation of staling, with fewer of the negative effects associated with endo-amylase activity. Reduction of endoamylase activity can lead to greater exospecificity, which can reduce branched dextrins and produce a higher quality bread.

The conversion of vegetable starches, especially corn starch, to ethanol is a rapidly expanding industry. Maltotetraose (G4 or DP4) syrup is one of many commercially important products derived from enzymatic treatment of starch. The conversion of vegetable starches, especially cornstarch, to maltotetraose and lower sugars, such as glucose or maltose, is a rapidly expanding industry.

The current process consists of two sequential enzyme-catalyzed steps that result in the production of glucose or maltose. Yeast can then be used to ferment the glucose to ethanol.

The first enzyme-catalyzed step is starch liquefaction. Typically, a starch suspension is gelatinized by rapid heating to 85°C or more. α -Amylases (EC 3.2.1.1) are used to degrade the viscous liquefact to maltodextrins. α -amylases are endohydrolases that catalyze the random cleavage of internal α -1,4-D-glucosidic bonds. As α -amylases break down the starch, the viscosity decreases. Because liquefaction typically is conducted at high temperatures, thermostable α -amylases, such as an α -amylase from *Bacillus* sp., are preferred for this step.

The maltodextrins produced in this manner generally cannot be fermented by yeast to form alcohol. A second enzyme-catalyzed saccharification step thus is required to break down the maltodextrins. Glucoamylases and/or maltogenic α -amylases commonly are used to catalyze the hydrolysis of non-reducing ends of the maltodextrins formed after liquefaction, releasing D-glucose, maltose and isomaltose. Debranching enzymes, such as pullulanases, can be used to aid saccharification. Saccharification typically takes place under acidic conditions at elevated temperatures, e.g., 60°C, pH 4.3.

G4 (also referred to as DP4) syrup has a number of advantageous properties compared to sucrose syrups. For example, partially replacing sucrose with G4 syrup in a food reduces the food's sweetness without affecting its taste or flavor. G4 syrup has high moisture retention in foods and exhibits less deleterious Maillard reaction products because of its lower glucose and maltose content. G4 syrup also has higher viscosity than sucrose, thus improving food texture. G4 syrup depresses the freezing point of water less than sucrose or high fructose syrup, so G4 syrup can better control the freezing points of frozen foods. After ingestion, G4

syrup also affects osmotic pressure less than sucrose. Together, these qualities make G4 syrup ideally suited as an ingredient in foods and medical products. G4 syrup is useful in other industries, as well. For example, G4 syrup imparts gloss and can be used advantageously as a paper sizer. See, e.g., Kimura et al., "Maltotetraose, a new saccharide of tertiary property," Starch 42: 151-57 (1990).

One of the yeasts used to produce ethanol is *Saccharomyces cerevisiae*. *S. cerevisiae* contains α -glucosidase that has been shown to utilize mono-, di-, and tri-saccharides as substrates. Yoon et al., Carbohydrate Res. 338: 1127-32 (2003). The ability of *S. cerevisiae* to utilize tri-saccharides can be improved by Mg^{2+} supplementation and over-expression of AGT1 permease (Stambuck et al., Lett. Appl. Microbiol. 43: 370-76 (2006)), over-expression of MTT1 and MTT1alt to increase maltotriose uptake (Dietvorst et al., Yeast 22: 775-88 (2005)), or expression of the maltase MAL32 on the cell surface (Dietvorst et al., Yeast 24: 27-38 (2007)). The saccharification step could be omitted altogether, if the liquefaction step produced sufficient levels of mono-, di-, or tri-saccharides and *S. cerevisiae* or its genetically modified variants were used for the fermentation step.

Pseudomonas saccharophila expresses a maltotetraose-forming maltotetraohydrolase (EC 3.2.1.60; G4-forming amylase; G4-amylase). The nucleotide sequence of the *P. saccharophila* gene encoding PS4 has been determined. Zhou et al., "Nucleotide sequence of the maltotetraohydrolase gene from *Pseudomonas saccharophila*," FEBS Lett. 255: 37-41 (1989); GenBank Acc. No. X16732. PS4 is expressed as a precursor protein with an N-terminal 21-residue signal peptide. The mature form of PS4, as set forth in SEQ ID NO: 10, contains 530 amino acid residues with a catalytic domain at the N-terminus and a starch binding domain at the C-terminus. PS4 displays both endo- and exo- α -amylase activity. Endo- α -amylase activity is useful for decreasing the viscosity of gelatinized starch, and exo- α -amylase activity is useful for breaking down maltodextrins to smaller saccharides.

SUMMARY OF THE INVENTION

This invention relates to polypeptides, specifically amylase polypeptides and nucleic acids encoding these, and their uses e.g. as non-maltogenic exoamylases in producing food products. The amylases of the present invention have been engineered to have more beneficial qualities. Specifically, the amylases of the current invention show an altered exospecificity and/or altered thermostability. In particular, the polypeptides are derived from polypeptides having non-maltogenic exoamylase activity, in particular, glucan 1,4- α -maltotetrahydrolase (EC 3.2.1.60) activity. In one aspect, the polypeptides as defined herein have an improved antistaling effect compared to the amylase of SEQ ID NO: 1.

In another aspect, the polypeptides as defined herein have an improved thermostability compared to the amylase of SEQ ID NO: 1.

A variant polypeptide as set out in the claims is provided. In a further aspect, the use of such a variant polypeptide, including in and as food additives, food products, bakery products, improver compositions, feed products including animal feeds, etc as set out in the claims, is provided. In yet a further aspect, nucleic acids which encode and which relate to variant polypeptides, as set out in the claims, are provided. Methods for producing such variant polypeptides, as well as other aspects, are also set out in the claims.

LEGENDS TO THE FIGURE

- 10 The accompanying drawings are incorporated in and constitute a part of this specification and illustrate various embodiments.

Fig. 1 shows firmness development of bread baked with pMS1776 (SEQ ID NO: 12), pMS2020 (SEQ ID NO: 14), pMS2022 (SEQ ID NO: 15) and pMS2062 (SEQ ID NO: 16) compared to bread baked with a composition comprising SEQ ID NO: 1.

- 15 Fig. 2 shows firmness development of bread baked with pMS1776 (SEQ ID NO: 12), pMS1934 (SEQ ID NO: 13), pMS2022 (SEQ ID NO: 15) and pMS2062 (SEQ ID NO: 16) compared to bread baked with a composition comprising SEQ ID NO: 1 and Novamyl 1500.

DETAILED DISCLOSURE OF THE INVENTION

In accordance with this detailed description, the following abbreviations and definitions apply.

- 20 It should be noted that as used herein, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an enzyme" includes a plurality of such enzymes, and reference to "the formulation" includes reference to one or more formulations and equivalents thereof known to those skilled in the art, and so forth.

- 25 In one aspect, a polypeptide having amylase activity comprising an amino acid sequence having

a) at least 65 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 88 or 205, and/or

b) at least 78 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 16, 48, 97, 105, 235, 240, 248, 266, 311, 347, 350, 362, 364, 369, 393, 395, 396, 400, 401, 403, 412 or 409, and/or

c) at least 78 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more of the following amino acid substitutions: 42K/A/V/N/I/H/F, 34Q, 100Q/K/N/R, 272D, 392 K/D/E/Y/N/Q/R/T/G or 399C/H, and/or

d) at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 44, 96, 204, 354 or 377, and/or

e) at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises the following amino acid substitution: 392S

with reference to the position numbering of the sequence shown as SEQ ID NO: 1, is provided.

In one aspect, a polypeptide having amylase activity comprising an amino acid sequence having at least 65% sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 88, or 205 and/or one or more of the following amino acid substitutions: 42K, 235H/K/R, 240E, 392 K/D/E/Y or 409E with reference to the position numbering of the sequence shown as SEQ ID NO: 1, is provided.

The present invention also encompasses the use of polypeptides having a degree of sequence identity or sequence homology with amino acid sequence(s) defined herein or with a polypeptide having the specific properties defined herein. The present invention encompasses, in particular, peptides having a degree of sequence identity with SEQ ID NO: 1, defined below, or homologues thereof. Here, the term "homologue" means an entity having sequence identity with the subject amino acid sequences or the subject nucleotide sequences, where the subject amino acid sequence preferably is SEQ ID NO: 1 and the subject nucleotide sequence preferably is SEQ ID NO: 52.

In one aspect, the homologous amino acid sequence and/or nucleotide sequence should provide and/or encode a polypeptide which retains the functional activity and/or enhances the activity of a polypeptide of SEQ ID NO: 1 or SEQ ID NO: 10, preferably the activity of a polypeptide of SEQ ID NO: 1.

- 5 In the present context, a homologous sequence is taken to include an amino acid sequence which may be at least 65%, 70%, 75%, 78%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%, identical to the subject sequence. Typically, the homologues will comprise the same active sites etc. as the subject amino acid sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Sequence identity comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs use complex comparison algorithms to align two or more sequences that best reflect the evolutionary events that might have led to the difference(s) between the two or more sequences. Therefore, these algorithms operate with a scoring system rewarding alignment of identical or similar amino acids and penalising the insertion of gaps, gap extensions and alignment of non-similar amino acids. The scoring system of the comparison algorithms include:

- 20 i) assignment of a penalty score each time a gap is inserted (gap penalty score),
- ii) assignment of a penalty score each time an existing gap is extended with an extra position (extension penalty score),
- iii) assignment of high scores upon alignment of identical amino acids, and
- iv) assignment of variable scores upon alignment of non-identical amino acids.

25 Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons.

The scores given for alignment of non-identical amino acids are assigned according to a scoring matrix also called a substitution matrix. The scores provided in such substitution matrices are reflecting the fact that the likelihood of one amino acid being substituted with another during evolution varies and depends on the physical/chemical nature of the amino acid to be substituted. For example, the likelihood of a polar amino acid being substituted

with another polar amino acid is higher compared to being substituted with a hydrophobic amino acid. Therefore, the scoring matrix will assign the highest score for identical amino acids, lower score for non-identical but similar amino acids and even lower score for non-identical non-similar amino acids. The most frequently used scoring matrices are the PAM
5 matrices (Dayhoff et al. (1978), Jones et al. (1992)), the BLOSUM matrices (Henikoff and Henikoff (1992)) and the Gonnet matrix (Gonnet et al. (1992)).

Suitable computer programs for carrying out such an alignment include, but are not limited to, Vector NTI (Invitrogen Corp.) and the ClustalV, ClustalW and ClustalW2 programs (Higgins DG & Sharp PM (1988), Higgins et al. (1992), Thompson et al. (1994), Larkin et al.
10 (2007). A selection of different alignment tools are available from the ExPASy Proteomics server at www.expasy.org. Another example of software that can perform sequence alignment is BLAST (Basic Local Alignment Search Tool), which is available from the webpage of National Center for Biotechnology Information which can currently be found at <http://www.ncbi.nlm.nih.gov/> and which was firstly described in Altschul et al. (1990) J. Mol.
15 Biol. **215**; 403-410.

Once the software has produced an alignment, it is possible to calculate % similarity and % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

In one embodiment, it is preferred to use the ClustalW software for performing sequence
20 alignments. Preferably, alignment with ClustalW is performed with the following parameters for pairwise alignment:

Substitution matrix:	Gonnet 250
Gap open penalty:	20
Gap extension penalty:	0.2
Gap end penalty:	None

ClustalW2 is for example made available on the internet by the European Bioinformatics Institute at the EMBL-EBI webpage www.ebi.ac.uk under tools – sequence analysis –

ClustalW2. Currently, the exact address of the ClustalW2 tool is www.ebi.ac.uk/Tools/clustalw2.

In another embodiment, it is preferred to use the program Align X in Vector NTI (Invitrogen) for performing sequence alignments. In one embodiment, Exp10 has is used with the default settings:

Gap opening penalty: 10

Gap extension penalty: 0.05

Gapseparation penalty range: 8

Score matrix: blosum62mt2

- 10 Thus, the present invention also encompasses the use of variants, homologues and derivatives of any amino acid sequence of a protein or polypeptide as defined herein, particularly those of SEQ ID NO: 1 or those of SEQ ID NO: 2, 4, 6, 8 or 10 defined below.

- 15 The sequences, particularly those of variants, homologues and derivatives of SEQ ID NO: 1, may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

- 20 The present invention also encompasses conservative substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) that may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-conservative substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

Conservative substitutions that may be made are, for example within the groups of basic amino acids (Arginine, Lysine and Histidine), acidic amino acids (glutamic acid and aspartic acid), aliphatic amino acids (Alanine, Valine, Leucine, Isoleucine), polar amino acids (Glutamine, Asparagine, Serine, Threonine), aromatic amino acids (Phenylalanine, Tryptophan and Tyrosine), hydroxyl amino acids (Serine, Threonine), large amino acids (Phenylalanine and Tryptophan) and small amino acids (Glycine, Alanine).

Replacements may also be made by unnatural amino acids include; α * and α -disubstituted* amino acids, N-alkyl amino acids*, lactic acid*, halide derivatives of natural amino acids such as trifluorotyrosine*, p-Cl-phenylalanine*, p-Br-phenylalanine*, p-I-phenylalanine*, L-allyl-glycine*, β -alanine*, L- α -amino butyric acid*, L- γ -amino butyric acid*, L- α -amino isobutyric acid*, L- ϵ -amino caproic acid#, 7-amino heptanoic acid*, L-methionine sulfone#, L-norleucine*, L-norvaline*, p-nitro-L-phenylalanine*, L-hydroxyproline#, L-thioprolin*, methyl derivatives of phenylalanine (Phe) such as 4-methyl-Phe*, pentamethyl-Phe*, L-Phe (4-amino)#, L-Tyr (methyl)*, L-Phe (4-isopropyl)*, L-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxyl acid)*, L-diaminopropionic acid # and L-Phe (4-benzyl)*. The notation * has been utilised for the purpose of the discussion above (relating to homologous or non-conservative substitution), to indicate the hydrophobic nature of the derivative whereas # has been utilised to indicate the hydrophilic nature of the derivative, #* indicates amphipathic characteristics.

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β -alanine residues. A further form of variation, involves the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α -carbon substituent group is on the residue's nitrogen atom rather than the α -carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ et al. (1992), Horwell DC. (1995).

In one embodiment, the variant polypeptide is a *Pseudomonas saccharophila* amylase (PS4) variant having the sequence shown in SEQ ID NO: 1 and having at least at least 65%, at least 70%, at least 75%, at least 78%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% amino acid sequence identity therewith.

In one aspect, preferably the sequence used in the present invention is in a purified form. The term "purified" means that a given component is present at a high level. The component is desirably the predominant active component present in a composition.

Definitions

- 5 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. The following terms are provided below.

- "Amylase" means an enzyme that is, among other things, capable of catalyzing the degradation of starch. An endo-acting amylase activity cleaves •-D-(1• 4) O-glycosidic
10 linkages within the starch molecule in a random fashion. In contrast, an exo-acting amylolytic activity cleaves a starch molecule from the non-reducing end of the substrate. "Endo-acting amylase activity," "endo-activity," "endo-specific activity," and "endo-specificity" are synonymous, when the terms refer to the variant polypeptides as defined in the claims. The same is true for the corresponding terms for exo-activity.
- 15 "maltotetraose-forming maltotetrahydrolase; EC 3.2.1.60; G4-forming amylase; G4-amylase and glucan 1,4-alpha-maltotetrahydrolase" may be used interchangeably".

In the present context,, "PS4" is used to designate the maltotetrahydrolase from *Pseudomonas saccharophila*.

- A "variant" or "variants" refers to either polypeptides or nucleic acids. The term "variant"
20 may be used interchangeably with the term "mutant." Variants include insertions, substitutions, transversions, truncations, and/or inversions at one or more locations in the amino acid or nucleotide sequence, respectively. The phrases "variant polypeptide", "polypeptide", "variant" and "variant enzyme" mean a polypeptide/protein that has an amino acid sequence that has been modified from the amino acid sequence of SEQ ID NO: 1. The
25 variant polypeptides include a polypeptide having a certain percent, e.g., 65%, 66%, 68%, 70%, 72%, 74%, 76%, 78%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, of sequence identity with SEQ ID NO: 1. As used herein, "parent enzymes," "parent sequence," "parent polypeptide" mean enzymes and polypeptides from which the variant polypeptides are based, e.g., SEQ ID NO: 1. A "parent nucleic acid" means a nucleic
30 acid sequence encoding the parent polypeptide. The signal sequence of a "variant" may be the same (such as SEQ ID NO: 11) or may differ from the signal sequence of the wild-type PS4. A variant may be expressed as a fusion protein containing a heterologous polypeptide.

For example, the variant can comprise a signal peptide of another protein or a sequence designed to aid identification or purification of the expressed fusion protein, such as a His-Tag sequence.

To describe the various variants that are contemplated to be encompassed by the present disclosure, the following nomenclature will be adopted for ease of reference. Where the substitution includes a number and a letter, e.g., 141P, then this refers to { position according to the numbering system/substituted amino acid} . Accordingly, for example, the substitution of an amino acid to proline in position 141 is designated as 141P. Where the substitution includes a letter, a number, and a letter, e.g., A141P, then this refers to { original amino acid/position according to the numbering system/substituted amino acid} . Accordingly, for example, the substitution of alanine with proline in position 141 is designated as A141P.

Where two or more substitutions are possible at a particular position, this will be designated by contiguous letters, which may optionally be separated by slash marks "/", e.g., G303ED or G303E/D.

Position(s) and amino acid substitutions referred to herein are listed with reference to the corresponding position and the corresponding amino acid of SEQ ID NO: 1. Equivalent positions in another sequence may be found by aligning this sequence with SEQ ID NO:1 to find an alignment with the highest percent identity and thereafter determining which amino acid aligns to correspond with an amino acid of a specific position of SEQ ID NO:1. Such alignment and use of one sequence as a first reference is simply a matter of routine for one of ordinary skill in the art.

"Variant nucleic acids" can include sequences that are complementary to sequences that are capable of hybridizing to the nucleotide sequences presented herein, in particular to SEQ ID NO:52. For example, a variant sequence is complementary to sequences capable of hybridizing under stringent conditions, e.g., 50°C and 0.2X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), to the nucleotide sequences presented herein, in particular to SEQ ID NO:52 (pMS 382 DNA) More particularly, the term variant encompasses sequences that are complementary to sequences that are capable of hybridizing under highly stringent conditions, e.g., 65°C and 0.1X SSC, to the nucleotide sequences presented herein, in particular to SEQ ID NO:52 (pMS 382 DNA) The melting point (T_m) of a variant nucleic acid may be about 1, 2, or 3°C lower than the T_m of the wild-type nucleic acid. The variant nucleic acids include a polynucleotide having a certain percent, e.g., 80%, 85%, 90%, 95%, or 99%, of sequence identity with the nucleic acid encoding SEQ ID NO: 1.

As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation.

"Isolated" means that the sequence is at least substantially free from at least one other component that the sequence is naturally associated and found in nature, e.g., genomic sequences.

"Purified" means that the material is in a relatively pure state, e.g., at least about 90% pure, at least about 95% pure, or at least about 98% pure.

"Thermostable" means the enzyme retains activity after exposure to elevated temperatures.

The thermostability of an enzyme may be measured by its half-life ($t_{1/2}$), where half of the enzyme activity is lost by the half-life. The half-life value is calculated under defined conditions by measuring the residual amylase activity. To determine the half-life of the enzyme, the sample is heated to the test temperature for 1-10 min, and activity is measured using a standard assay for amylase activity, such as the Betamyl® assay (Megazyme, Ireland).

As used herein, "optimum pH" means the pH at which the variant polypeptides disclosed herein displays the activity in a standard assay for amylase activity, measured over a range of pH's.

As used herein, "polypeptide" is used interchangeably with the terms "amino acid sequence", "enzyme", "peptide" and/or "protein." As used herein, "nucleotide sequence" or "nucleic acid sequence" refers to an oligonucleotide sequence or polynucleotide sequence and variants, homologues, fragments and derivatives thereof. The nucleotide sequence may be of genomic, synthetic or recombinant origin and may be double-stranded or single-stranded, whether representing the sense or anti-sense strand. As used herein, the term "nucleotide sequence" includes genomic DNA, cDNA, synthetic DNA, and RNA.

"Homologue" means an entity having a certain degree of identity or "homology" with the subject amino acid sequences and the subject nucleotide sequences. A "homologous sequence" includes a polynucleotide or a polypeptide having a certain percent, e.g., 80%, 85%, 90%, 95%, or 99%, of sequence identity with another sequence. Percent identity means that, when aligned, that percentage of bases or amino acid residues are the same when comparing the two sequences. Amino acid sequences are not identical, where an amino acid is substituted, deleted, or added compared to the subject sequence. The percent sequence identity typically is measured with respect to the mature sequence of the subject

protein, i.e., following removal of a signal sequence, for example. Typically, homologues will comprise the same active site residues as the subject amino acid sequence. Homologues also retain amylase activity, although the homologue may have different enzymatic properties than the wild-type PS4.

- 5 As used herein, "hybridization" includes the process by which a strand of nucleic acid joins with a complementary strand through base pairing, as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies. The variant nucleic acid may exist as single- or double-stranded DNA or RNA, an RNA/DNA heteroduplex or an RNA/DNA copolymer. As used herein, "copolymer" refers to a single nucleic acid strand that comprises
10 both ribonucleotides and deoxyribonucleotides. The variant nucleic acid may be codon-optimized to further increase expression.

As used herein, a "synthetic" compound is produced by in vitro chemical or enzymatic synthesis. It includes, but is not limited to, variant nucleic acids made with optimal codon usage for host organisms, such as a yeast cell host or other expression hosts of choice.

- 15 As used herein, "transformed cell" includes cells, including both bacterial and fungal cells, which have been transformed by use of recombinant DNA techniques. Transformation typically occurs by insertion of one or more nucleotide sequences into a cell. The inserted nucleotide sequence may be a heterologous nucleotide sequence, i.e., is a sequence that is not natural to the cell that is to be transformed, such as a fusion protein.
- 20 As used herein, "operably linked" means that the described components are in a relationship permitting them to function in their intended manner. For example, a regulatory sequence operably linked to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

- As used herein the term "starch" refers to any material comprised of the complex
25 polysaccharide carbohydrates of plants, such as corn, comprised of amylose and amylopectin with the formula $(C_6H_{10}O_5)_x$, where X can be any number. The term "granular starch" refers to raw, i.e., uncooked starch, e.g., starch that has not been subject to gelatinization.

- The term "liquefaction" refers to the stage in starch conversion in which gelatinized starch is hydrolyzed to give low molecular weight soluble dextrins. As used herein the term
30 "saccharification" refers to enzymatic conversion of starch to glucose. The term "degree of polymerization" (DP) refers to the number (n) of anhydroglucopyranose units in a given saccharide. Examples of DP1 are the monosaccharides glucose and fructose. Examples of DP2 are the disaccharides maltose and sucrose.

As used herein the term "dry solids content" (ds) refers to the total solids of a slurry in a dry weight percent basis. The term "slurry" refers to an aqueous mixture containing insoluble solids.

The phrase "simultaneous saccharification and fermentation (SSF)" refers to a process in the production of biochemicals in which a microbial organism, such as an ethanol producing microorganism and at least one enzyme, such as PS4 or a variant thereof, are present during the same process step. SSF refers to the contemporaneous hydrolysis of granular starch substrates to saccharides and the fermentation of the saccharides into alcohol, for example, in the same reactor vessel.

As used herein "ethanologenic microorganism" refers to a microorganism with the ability to convert a sugar or oligosaccharide to ethanol.

1.2. Abbreviations

The following abbreviations apply unless indicated otherwise:

15	ADA	azodicarbonamide
	cDNA	complementary DNA
	CGTase	cyclodextrin glucanotransferase
	DEAE	diethylamino ethanol
	dH ₂ O	deionized water
	DNA	deoxyribonucleic acid
20	ds-DNA	double-stranded DNA
	EC	enzyme commission for enzyme classification
	FGSC	Fungal Genetics Stock Center
	G121F	glycine (G) residue at position 121 of SEQ ID NO: 2 is replaced with a phenylalanine (F) residue, where amino acids are designated by single letter abbreviations commonly known in the art
25		
	HPLC	High Performance Liquid Chromatography
	mRNA	messenger ribonucleic acid
	PCR	polymerase chain reaction
30	PDB	Protein Database Base
	PEG	polyethyleneglycol
	ppm	parts per million
	PS4	<i>P. saccharophila</i> G4-forming amylase
	RT-PCR	reverse transcriptase polymerase chain reaction
35	SAS	<i>P. saccharophila</i> G4-forming amylase
	SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
	1X SSC	0.15 M NaCl, 0.015 M sodium citrate, pH 7.0
	SSF	simultaneous saccharification and fermentation
	t _{1/2}	half life
40	T _m	melting temperature (°C) at which 50% of the subject protein is melted
	• T _m	°C increase in the T _m
	w/v	weight/volume
	w/w	weight/weight

2. Polypeptide variants of SEQ ID NO: 1

In one aspect, a polypeptide having a substitution at one or more positions which effect an altered property, which may be any combination of altered exospecificity, endospecificity or altered thermostability, or an altered handling property, relative to SEQ ID NO: 1, is provided. Such variant polypeptides are also referred to in this document for convenience as "variant polypeptide", "polypeptide variant" or "variant". In one aspect, the polypeptides as defined herein have an improved antistaling effect compared to the amylase of SEQ ID NO: 1. In another aspect, the polypeptides as defined herein have an improved thermostability compared to the amylase of SEQ ID NO: 1. In highly preferred embodiments, they have the added advantage of higher thermostability, or higher exoamylase activity or higher pH stability, or any combination.

In one aspect, the variants as defined herein exhibit enzyme activity. In one aspect, the variant polypeptides comprise amylase activity. In a further aspect, the variant polypeptides comprise exoamylase activity. In a further aspect, the variant polypeptides exhibit non-maltogenic exoamylase activity. In one aspect, the variants as defined herein are derivable from *Pseudomonas saccharophila* α -amylase (PS4). In one aspect, the variants as defined herein are a maltotetraose-forming maltotetrahydrolase, also called EC 3.2.1.60; G4-forming amylase; G4-amylase or glucan 1,4- α -maltotetrahydrolase.

Compositions, including food additives, food products, bakery products, improver compositions, feed products including animal feeds, etc comprising such variant polypeptides as defined herein, such as those which have non-maltogenic exoamylase activity, as well as methods of making and using such polypeptides and the compositions, are provided herein.

As noted above, the variant polypeptides may comprise one or more improved handling properties, preferably improved baking properties. Thus, the variant polypeptides are such that the food products so treated have one or more of (preferably all of) a lower firmness, a higher resilience, a higher cohesiveness, a lower crumbliness or a higher foldability. Such improved handling or baking properties exhibited by the variant polypeptides are described in further detail below.

Furthermore a treatment of food products, particularly doughs and bakery products with such polypeptides, and such that the food products exhibit the desired qualities set out above, is provided.

Further is provided other uses of the variants described herein and compositions comprising these variants, such as in the preparation of detergents, as sweeteners, syrups, etc. The compositions include the polypeptide together with at least one other component. In particular, food or feed additives comprising the polypeptides, are provided.

- 5 An isolated and/or purified polypeptide comprising a variant polypeptide as defined herein is provided. In one embodiment, the variant polypeptide is a mature form of the polypeptide (SEQ ID NO: 1), wherein the 21 amino acid leader sequence is cleaved, so that the N-terminus of the polypeptide begins at the aspartic acid (D) residue. In one aspect, the variants include a C-terminal starch binding domain. A representative amino acid sequence
10 of a starch binding domain comprises or consist of an amino acid sequence of SEQ ID NO: 36. Other variants include variants wherein between one and about 25 amino acid residues have been added or deleted with respect to SEQ ID NO: 1. In one aspect, the variant has the amino acid sequence of SEQ ID NO: 1, wherein any number between one and about 25 amino acids have been substituted. In a further aspect, the variant has the amino acid
15 sequence of SEQ ID NO: 1, wherein any number between three and twelve amino acids has been substituted. In a further aspect, the variant has the amino acid sequence of SEQ ID NO: 1, wherein any number between five and nine amino acids has been substituted.

In one aspect, at least at least two, in another aspect at least three, and yet in another aspect at least five amino acids of SEQ ID NO: 1 has been substituted.

- 20 In a further aspect, the length of the polypeptide variant is 390 to 540 amino acids. In a further aspect, the length of the polypeptide variant is 410 to 440 amino acids. In a further aspect, the length of the polypeptide variant is 420 to 435 amino acids. In a further aspect, the length of the polypeptide variant is 429 to 430 amino acids.

- In one aspect, the invention relates to a polypeptide having amylase activity comprising an
25 amino acid sequence having:

- a) at least 65 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 88 or 205, and/or
- b) at least 78 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and
30 wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 16, 48, 97, 105, 235, 240, 248, 266, 311, 347, 350, 362, 364, 369, 393, 395, 396, 400, 401, 403, 412 or 409, and/or

c) at least 78 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more of the following amino acid substitutions: 42K/A/V/N/I/H/F, 34Q, 100Q/K/N/R, 272D, 392 K/D/E/Y/N/Q/R/T/G or 399C/H, and/or

5 d) at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 44, 96, 204, 354 or 377, and/or

e) at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises the following amino acid substitution: 392S

10 wherein an amino acid substitution is relative to the corresponding amino acid of SEQ ID NO: 1 and the positions are with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

In a further aspect, the polypeptide as defined herein comprises one or more amino acid substitutions at the following positions: 88, 205, 235, 240, 248, 266, 311, 377 or 409 and/or
 15 one or more of the following amino acid substitutions: 42K/A/V/N/I/H/F, 34Q, 100Q/K/N/R, 272D or 392K/D/E/Y/N/Q/R/S/T/G. In a further aspect, the polypeptide as defined herein comprises one or more amino acid substitutions at the following positions: 88, 205, 235, 240, 311 or 409 and/or one or more of the following amino acid substitutions: 42K/N/I/H/F, 272D, or 392 K/D/E/Y/N/Q/R/S/T/G. In a further aspect, the polypeptide as defined herein
 20 comprises amino acid substitutions at least in four, five or in all of the following positions: 88, 205, 235, 240, 311 or 409 and/or has at least one, or two the following amino acid substitutions: 42K/N/I/H/F, 272D or 392 K/D/E/Y/N/Q/R/S/T/G. In a further aspect, the polypeptide as defined herein further comprises one or more of the following amino acids 33Y, 34N, 70D, 121F, 134R, 141P, 146G, 157L, 161A, 178F, 179T, 223E/S/K/A, 229P, 307K,
 25 309P and 334P. In a further aspect, the polypeptide as defined herein further has the following amino acids 33Y, 34N, 70D, 121F, 134R, 141P, 146G, 157L, 161A, 178F, 179T, 229P, 307K, 309P and 334P. In a further aspect, the polypeptide as defined herein has at least one of the amino acid substitutions in a) of claim 1 and further comprises one or more amino acid substitutions at the following positions: 44, 96, 204, 354 or 377. In a further
 30 aspect, the polypeptide as defined herein has at least 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 1. In a further aspect, the polypeptide as defined herein has the amino acid 34Q. In a further aspect, the polypeptide as defined herein has the amino acid 42K/F/H/I/N/A/V. In a further aspect, the polypeptide as defined herein has the amino acid 42K/F/H/I/N. In a further aspect, the polypeptide as
 35 defined herein has the amino acid 42K. In a further aspect, the polypeptide as defined herein

comprises an amino acid substitution in position 88. In a further aspect, the polypeptide as defined herein has the amino acid 88L/Y/K. In a further aspect, the polypeptide as defined herein has the amino acid 88L. In a further aspect, the polypeptide as defined herein has the amino acid 88Y. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 205. In a further aspect, the polypeptide as defined herein has the amino acid 205L/K/M/N/Q/R/V/Y. In a further aspect, the polypeptide as defined herein has the amino acid 205L/K/M/N/Q/R/V. In a further aspect, the polypeptide as defined herein has the amino acid 205L. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 235. In a further aspect, the polypeptide as defined herein has the amino acid 235H/K/R/Q/S. In a further aspect, the polypeptide as defined herein has the amino acid 235H/K/R/Q. In a further aspect, the polypeptide as defined herein has the amino acid 235H/K/R. In a further aspect, the polypeptide as defined herein has the amino acid 235R. In a further aspect, the polypeptide as defined herein has the amino acid 235H. In a further aspect, the polypeptide as defined herein has the amino acid 235K. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 240. In a further aspect, the polypeptide as defined herein has the amino acid 240E/H/M/D/S. In a further aspect, the polypeptide as defined herein has the amino acid 240E/H/M/D. In a further aspect, the polypeptide as defined herein has the amino acid 240E. In a further aspect, the polypeptide as defined herein has the amino acid 272D. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 311. In a further aspect, the polypeptide as defined herein has the amino acid 311P. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 409. In a further aspect, the polypeptide as defined herein has the amino acid 409H/Q/T/E. In a further aspect, the polypeptide as defined herein has the amino acid 409E. In a further aspect, the polypeptide as defined herein has the amino acid 100Q/K/N/R. In a further aspect, the polypeptide as defined herein has the amino acid 100Q. In a further aspect, the polypeptide as defined herein has the amino acid 392D/E/K/Y/N/Q/R/S/T/G. In a further aspect, the polypeptide as defined herein has the amino acid 392D/E/K/Y/N/Q/R/S/T. In a further aspect, the polypeptide as defined herein has the amino acid 392D/E/K/Y. In a further aspect, the polypeptide as defined herein has the amino acid 392D. In a further aspect, the polypeptide as defined herein has the amino acid 392E. In a further aspect, the polypeptide as defined herein has the amino acid 392K. In a further aspect, the polypeptide as defined herein has the amino acid 392Y. In a further aspect, the polypeptide as defined herein has both of the following amino acids 235R and 311P. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 16. In a further aspect, the polypeptide as defined herein has the amino acid 16/A/E/K. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 48. In a further aspect, the polypeptide as defined herein has the

amino acid 48/C/L. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 97. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 105. In a further aspect, the polypeptide as defined herein has the amino acid 105/N/R. In a further aspect, the polypeptide as defined
5 herein comprises an amino acid substitution in position 248. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 266. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 347. In a further aspect, the polypeptide as defined herein has the amino acid 347/C/D/H/K. In a further aspect, the polypeptide as defined herein comprises an amino acid
10 substitution in position 350. In a further aspect, the polypeptide as defined herein has the amino acid 350E/H/N. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 354. In a further aspect, the polypeptide as defined herein has the amino acid 354D/E. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 362. In a further aspect, the polypeptide as defined
15 herein has the amino acid 362E/H/P. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 364. In a further aspect, the polypeptide as defined herein has the amino acid 364E/K/NQ. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 369. In a further aspect, the polypeptide as defined herein has the amino acid 369I/N. In a further aspect, the polypeptide
20 as defined herein comprises an amino acid substitution in position 377. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 393. In a further aspect, the polypeptide as defined herein has the amino acid 393D/E/K. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 395. In a further aspect, the polypeptide as defined herein has the amino acid 395C/E/K. In a
25 further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 396. In a further aspect, the polypeptide as defined herein has the amino acid 396D/E. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 399. In a further aspect, the polypeptide as defined herein has the amino acid 399C/H. In a further aspect, the polypeptide as defined herein comprises an
30 amino acid substitution in position 400. In a further aspect, the polypeptide as defined herein has the amino acid 400S/W. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 401. In a further aspect, the polypeptide as defined herein has the amino acid 401D/K. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 403. In a further aspect, the polypeptide as
35 defined herein has the amino acid 403E/T/V. In a further aspect, the polypeptide as defined herein comprises an amino acid substitution in position 412. In a further aspect, the polypeptide as defined herein has the amino acid 412D/N. In a further aspect, the polypeptide as defined herein further comprises one or more of the following amino acids 121F, 134R, 141P, 229P, or 307K. In a further aspect, the polypeptide as defined herein has

one or more of the following amino acids 42K, 88L, 205L, 235R, 240E, 272D, 311P, 392D, or 409E. In a further aspect, the polypeptide as defined herein comprises the following amino acids 42K, 88L, 235R, 311P, 392D and either 223S, 223K or 223A. In a further aspect, the polypeptide as defined herein further comprises one or more selected from the group consisting of 272D, 409E, 205L and 240E. In a further aspect, the polypeptide as defined herein has at least four, five, six, seven or eight of the following amino acids 42K, 88L, 205L, 235R, 240E, 272D, 311P, 392D, or 409E. In a further aspect, the polypeptide as defined herein has the amino acid 223A/K/S. In a further aspect, the polypeptide as defined herein has the following amino acids 42K, 88L, 223S, 235R, 311P and 392D. In a further aspect, the polypeptide as defined herein has the following amino acids 42K, 88L, 223K, 235R, 272D, 311P and 392D. In a further aspect, the polypeptide as defined herein has the following amino acids 42K, 88L, 223S, 235R, 311P, 392D and 409E. In a further aspect, the polypeptide as defined herein has the following amino acids 42K, 88L, 205L, 223S, 235R, 311P, 392D and 409E. In a further aspect, the polypeptide as defined herein has the following amino acids 42K, 88L, 205L, 223K, 240E, 235R, 311P, 392D and 409E. In a further aspect, the polypeptide as defined herein has the following amino acids 42K, 88L, 205L, 223A, 235R, 311P, 392D and 409E. In a further aspect, the polypeptide as defined herein has one or more amino acids in the N-terminus. In one aspect, the polypeptide as defined herein has the amino acid M at the N-terminus. In a further aspect, the polypeptide as defined herein comprises one or more amino acid substitutions at the following positions: 88, 205, 235 or 409 and/or one or more of the following amino acid substitutions: 42K, 34Q, 100Q, 223A/S, 240E, 311P, 392D, or 409E with reference to the position numbering of the sequence shown as SEQ ID NO: 1. In a further aspect, the polypeptide as defined herein comprises one or more of the following amino acid substitutions: 42K, 34Q, 88K/L/Y, 100Q, 205L, 223A/S/K, 235K/H/Q/R, 240E/D, 248H, 266T, 311P, 377D/E/P, 392K/D/Y or 409E. In a further aspect, the polypeptide as defined herein comprises one or more of the following amino acid substitutions: 34Q, 42K, 88L, 100Q, 205L, 223A/S/K, 235K/R, 240E, 311P, 392D or 409E. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 235R and 311P. In a further aspect, the polypeptide as defined herein comprises the amino acid substitution 42K. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 42K and 223S. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 42K, 223S and 392D. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 42K, 88L, 223A, 235R, 311P and 392D. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 42K, 88L, 205L, 223S, 235R, 240E, 311P, 392D and 409E. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 42K, 88L, 205L, 223K, 235R, 240E, 311P, 392D and 409E. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 42K, 88L, 205L, 223S,

235R, 311P and 392D. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 34Q, 42K, 88L, 205L, 223K, 235R, 240E, 311P, 392D and 409E. In a further aspect, the polypeptide as defined herein comprises the following amino acid substitutions: 42K, 88L, 100Q, 205L, 223K, 235R, 240E, 311P, 392D and 409E.

- 5 Representative embodiments of the polypeptides as defined herein comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33 and SEQ ID NO: 34. Further representative
- 10 embodiments of the polypeptides as defined herein comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17. Further representative embodiments of the polypeptides as defined herein has an amino acid sequence selected from the group
- 15 consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33 and SEQ ID NO: 34. Further representative embodiments of the polypeptides as
- 20 defined herein has an amino acid sequence selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17, and optionally one or more amino acids at the N-terminus.

In one aspect, the polypeptides as defined herein comprises the sequence HGGDEIILQFHWN (SEQ ID NO: 37) at the positions corresponding to positions 13-26 with reference to the

25 position numbering of the sequence shown as SEQ ID NO: 1.

In one aspect, the polypeptides as defined herein comprises the sequence DGF-X1-AIW-X2-P-X3-PWRD-X4-SSW (SEQ ID NO: 38), wherein X1 is S or T, X2 is M or L, X3 is V or P and X4 is any natural occurring amino acid residue, preferably an L-amino acid, at the positions

30 corresponding to positions 49-66 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

In one aspect, the polypeptides as defined herein comprises the sequence GGEGYFW (SEQ ID NO: 39) at the positions corresponding to positions 79-85 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

In one aspect, the polypeptides as defined herein comprises the sequence VPNH (SEQ ID NO: 40) at the positions corresponding to positions 114-117 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

5 In one aspect, the polypeptides as defined herein comprises the sequence CDDGD (SEQ ID NO: 41) at the positions corresponding to positions 150-154 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

In one aspect, the polypeptides as defined herein comprises the sequence AGGFRFDFVRG (SEQ ID NO: 42) at the positions corresponding to positions 187-197 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

10 In one aspect, the polypeptides as defined herein comprises the sequence FALK (SEQ ID NO: 43) at the positions corresponding to positions 256-259 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

15 In one aspect, the polypeptides as defined herein comprises the sequence WREVAVTFVDNHD (SEQ ID NO: 44) at the positions corresponding to positions 282-294 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

In one aspect, the polypeptides as defined herein comprises the sequence GYSPG (SEQ ID NO: 45) at the positions corresponding to positions 296-300 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

20 In one aspect, the polypeptides as defined herein comprises the sequence GQH (SEQ ID NO: 46) at the positions corresponding to positions 304-306 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

In one aspect, the polypeptides as defined herein comprises the sequence AYAYI (SEQ ID NO: 47) at the positions corresponding to positions 318-322 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

25 In one aspect, the polypeptides as defined herein comprises the sequence SPGTP (SEQ ID NO: 48) at the positions corresponding to positions 325-329 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

In one aspect, the polypeptides as defined herein comprises the sequence VYW (SEQ ID NO: 49) at the positions corresponding to positions 331-333 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

In one aspect, the polypeptides as defined herein comprises the sequence HMYDWG (SEQ ID NO: 50) at the positions corresponding to positions 335-340 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

In another aspect, the polypeptide variant as defined herein has the sequence of SEQ ID NO:1, wherein any number between one and about 25 amino acids have been substituted. Representative examples of polypeptide variants as defined herein having single or several amino acid substitutions are shown in below TABLE A:

pMS	Mutations compared to pMS382 having SEQ ID NO:1
pms1104 (SEQ ID NO: 20)	Q42K
pms1105 (SEQ ID NO: 27)	Q42K, E223S
pms1723 (SEQ ID NO: 28)	Q42K, E223S, A392D
pms2104 (SEQ ID NO: 29)	Q42K, R88L, E223A, T235R, Q311P, A392D
pms2118 (SEQ ID NO: 34)	Q42K, R88L, S205L, E223S, T235R, Q240E, Q311P, A392D, S409E
pms2124 (SEQ ID NO: 31)	Q42K, R88L, S205L, E223K, T235R, Q240E, Q311P, A392D, S409E
pms1284 (SEQ ID NO: 23)	T235R, Q311P
pms1286 (SEQ ID NO: 22)	T235R
pms1290 (SEQ ID NO: 24)	T235K
pms465 (SEQ ID NO: 18)	E223S
pms1042 (SEQ ID NO: 19)	Q311P
pms1153 (SEQ ID NO: 21)	R88L
pms1484 (SEQ ID NO: 25)	S205L
pms1579 (SEQ ID NO: 26)	S409E
pms2138 (SEQ ID NO: 30)	Q42K, R88L, S205L, E223S, T235R, Q311P, A392D
pms2177 (SEQ ID NO: 32)	N34Q, Q42K, R88L, S205L, E223K, T235R, Q240E, Q311P, A392D, S409E
pms2178 (SEQ ID NO: 33)	Q42K, R88L, G100Q, S205L, E223K, T235R, Q240E, Q311P, A392D, S409E
pms1776 (SEQ ID NO: 12)	Q42K, R88L, E223S, T235R, Q311P, A392D
pms1934 (SEQ ID NO: 13)	Q42K, R88L, E223K, T235R, H272D, Q311P, A392D

pMS	Mutations compared to pMS382 having SEQ ID NO:1
pms2020 (SEQ ID NO: 14)	Q42K, R88L, E223S, T235R, Q311P, A392D, S409E
pms2022 (SEQ ID NO: 15)	Q42K, R88L, S205L, E223S, T235R, Q311P, A392D, S409E
pms2062 (SEQ ID NO: 16)	Q42K, R88L, S205L, E223K, Q240E, T235R, Q311P, A392D, S409E
pms2171 (SEQ ID NO: 17)	Q42K, R88L, S205L, E223A, T235R, Q311P, A392D, S409E

The polypeptide variants as defined herein may have an altered thermostability, an altered endo-amylase activity, an altered exo-amylase activity, and/or an altered ratio of exo- to endo amylase activity compared to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 10. In one aspect, the polypeptides as defined herein have an improved antistaling effect compared to the amylase of SEQ ID NO: 1. In another aspect, the polypeptides as defined herein have an improved thermostability compared to the amylase of SEQ ID NO: 1.

The polypeptide variants as defined herein may have up to 25, 23, 21, 19, 17, 15, 13, 11, 9, 8, 7, 6, 5 amino acid deletions, additions, insertions, or substitutions compared to the amino acid sequence of SEQ ID NO: 1.

The present disclosure also relates to each and every G4-amylase backbone having SEQ ID NO: 2, 4, 6, or 8 comprising the substitutions as further defined herein.

Nucleic acids encoding the polypeptide variants above also are provided. In one embodiment, a nucleic acid encoding a polypeptide variant as defined herein is a nucleotide sequence encoding the protein comprising an amino acid sequence of residues 1-429 of SEQ ID NO: 1 as set forth in SEQ ID NO: 52. For example nucleotide sequence SEQ ID NO: 3 encodes the G4 amylase backbone with SEQ ID NO: 2. As is well understood by one skilled in the art, the genetic code is degenerate, meaning that multiple codons in some cases may encode the same amino acid. Nucleic acids can include genomic DNA, mRNA, and cDNA that encodes a polypeptide variant

2.1. Polypeptide variant characterization

Enzyme variants can be characterized by their nucleic acid and primary polypeptide sequences, by three dimensional structural modeling, and/or by their specific activity.

Additional characteristics of the polypeptide variants as defined herein include stability, pH range, oxidation stability, and thermostability, for example. Levels of expression and enzyme

activity can be assessed using standard assays known to the artisan skilled in this field. In another aspect, variants demonstrate improved performance characteristics relative to the polypeptide with SEQ ID NO: 1, such as improved stability at high temperatures, e.g., 65-85°C. In one aspect, the polypeptide variants as defined herein are advantageous for use in liquefaction or other processes that require elevated temperatures, such as baking. For example, a thermostable polypeptide variant as defined herein can degrade starch at temperatures of about 55°C to about 85°C or more.

An expression characteristic means an altered level of expression of the variant, when the variant is produced in a particular host cell. Expression generally relates to the amount of active variant that is recoverable from a fermentation broth using standard techniques known in this art over a given amount of time. Expression also can relate to the amount or rate of variant produced within the host cell or secreted by the host cell. Expression also can relate to the rate of translation of the mRNA encoding the variant enzyme.

A nucleic acid complementary to a nucleic acid encoding any of the polypeptide variants as defined herein set forth herein is provided. Additionally, a nucleic acid capable of hybridizing to the complement is provided. In another embodiment, the sequence for use in the methods and compositions described here is a synthetic sequence. It includes, but is not limited to, sequences made with optimal codon usage for expression in host organisms, such as yeast.

3. Production of the polypeptide variants as defined herein

The polypeptide variants as provided herein may be produced synthetically or through recombinant expression in a host cell, according to procedures well known in the art. The expressed polypeptide variant as defined herein optionally is isolated prior to use. In another embodiment, the polypeptide variant as defined herein is purified following expression.

Methods of genetic modification and recombinant production of polypeptide variants are described, for example, in U.S. Patent Nos. 7,371,552, 7,166,453; 6,890,572; and 6,667,065; and U.S. Published Application Nos. 2007/0141693; 2007/0072270; 2007/0020731; 2007/0020727; 2006/0073583; 2006/0019347; 2006/0018997; 2006/0008890; 2006/0008888; and 2005/0137111. The relevant teachings of these disclosures, including polypeptide-encoding polynucleotide sequences, primers, vectors, selection methods, host cells, purification and reconstitution of expressed polypeptide variants, and characterization of polypeptide variants as defined herein, including useful buffers, pH ranges, Ca²⁺ concentrations, substrate concentrations and enzyme concentrations for enzymatic assays, are herein incorporated by reference.

In another embodiment, suitable host cells include a Gram positive bacterium selected from the group consisting of *Bacillus subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus*, *B. thuringiensis*, *Streptomyces lividans*, or *S. murinus*; or a Gram negative bacterium, wherein said Gram negative bacterium is *Escherichia coli* or a *Pseudomonas* species. In one aspect, the host cell is a *B. subtilis* or *B. licheniformis*. In one embodiment, the host cell is *B. subtilis*, and the expressed protein is engineered to comprise a *B. subtilis* signal sequence, as set forth in further detail below. In one aspect, the host cell expresses the polynucleotide as set out in the claims.

In some embodiments, a host cell is genetically engineered to express a polypeptide variant as defined herein with an amino acid sequence having at least about 78%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity with the polypeptide of SEQ ID NO: 1. In some embodiments, the polynucleotide encoding a polypeptide variant as defined herein will have a nucleic acid sequence encoding the protein of SEQ ID NO: 1 or a nucleic acid sequence having at least about 78%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleic acid encoding the protein of SEQ ID NO: 1. In one embodiment, the nucleic acid sequence has at least about 78%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the nucleic acid of SEQ ID NO: 52.

3.1. Vectors

In one aspect, the invention relates to a vector comprising a polynucleotide. In one aspect, of the invention a bacterial cell comprises the vector. In some embodiments, a DNA construct comprising a nucleic acid encoding a variant is transferred to a host cell in an expression vector that comprises regulatory sequences operably linked to an encoding sequence. The vector may be any vector that can be integrated into a fungal host cell genome and replicated when introduced into the host cell. The FGSC Catalogue of Strains, University of Missouri, lists suitable vectors. Additional examples of suitable expression and/or integration vectors are provided in Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001); Bennett et al., *MORE GENE MANIPULATIONS IN FUNGI*, Academic Press, San Diego (1991), pp. 396-428; and U.S. Patent No. 5,874,276. Exemplary vectors include pFB6, pBR322, PUC18, pUC100 and pENTR/D, pDONTM201, pDONRTM221, pENTRTM, pGEM[®]3Z and pGEM[®]4Z. Exemplary for use in bacterial cells include pBR322 and pUC19, which permit replication in *E. coli*, and pE194, for example, which permits replication in *Bacillus*.

In some embodiments, a nucleic acid encoding a variant is operably linked to a suitable promoter, which allows transcription in the host cell. The promoter may be derived from

genes encoding proteins either homologous or heterologous to the host cell. Suitable non-limiting examples of promoters include *cbh1*, *cbh2*, *egl1*, and *egl2* promoters. In one embodiment, the promoter is one that is native to the host cell. For example, when *P. saccharophila* is the host, the promoter is a native *P. saccharophila* promoter. An "inducible promoter" is a promoter that is active under environmental or developmental regulation. In another embodiment, the promoter is one that is heterologous to the host cell.

In some embodiments, the coding sequence is operably linked to a DNA sequence encoding a signal sequence. A representative signal peptide is SEQ ID NO: 11, which is the native signal sequence of the PS4 precursor. In other embodiments, the DNA encoding the signal sequence is replaced with a nucleotide sequence encoding a signal sequence from a species other than *P. saccharophila*. In this embodiment, the polynucleotide that encodes the signal sequence is immediately upstream and in-frame of the polynucleotide that encodes the polypeptide. The signal sequence may be selected from the same species as the host cell. In one non-limiting example, the signal sequence is a cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) signal sequence from *Bacillus* sp., and the variant polypeptides disclosed herein is expressed in a *B. subtilis* host cell. A methionine residue may be added to the N-terminus of the signal sequence.

In additional embodiments, a signal sequence and a promoter sequence comprising a DNA construct or vector to be introduced into a fungal host cell are derived from the same source. In some embodiments, the expression vector also includes a termination sequence. In one embodiment, the termination sequence and the promoter sequence are derived from the same source. In another embodiment, the termination sequence is homologous to the host cell.

In some embodiments, an expression vector includes a selectable marker. Examples of suitable selectable markers include those that confer resistance to antimicrobial agents, e.g., hygromycin or phleomycin. Nutritional selective markers also are suitable and include *amdS*, *argB*, and *pyr4*. In one embodiment, the selective marker is the *amdS* gene, which encodes the enzyme acetamidase; it allows transformed cells to grow on acetamide as a nitrogen source. The use of an *A. nidulans* *amdS* gene as a selective marker is described in Kelley et al., EMBO J. 4: 475-479 (1985) and Penttila et al., Gene 61: 155-164 (1987).

A suitable expression vector comprising a DNA construct with a polynucleotide encoding a variant may be any vector that is capable of replicating autonomously in a given host organism or integrating into the DNA of the host. In some embodiments, the expression vector is a plasmid. In some embodiments, two types of expression vectors for obtaining expression of genes are contemplated. The first expression vector comprises DNA sequences

in which the promoter, coding region, and terminator all originate from the gene to be expressed. In some embodiments, gene truncation is obtained by deleting undesired DNA sequences, e.g., DNA encoding the C-terminal starch-binding domain, to leave the domain to be expressed under control of its own transcriptional and translational regulatory sequences.

5 The second type of expression vector is preassembled and contains sequences required for high-level transcription and a selectable marker. In some embodiments, the coding region for a gene or part thereof is inserted into this general-purpose expression vector, such that it is under the transcriptional control of the expression construct promoter and terminator sequences. In some embodiments, genes or part thereof are inserted downstream of the
10 strong *cbh1* promoter.

3.2. Transformation, expression and culture of host cells

Introduction of a DNA construct or vector into a host cell includes techniques such as transformation; electroporation; nuclear microinjection; transduction; transfection, e.g., lipofection mediated and DEAE-Dextrin mediated transfection; incubation with calcium
15 phosphate DNA precipitate; high velocity bombardment with DNA-coated microprojectiles; and protoplast fusion. General transformation techniques are known in the art. See, e.g., Ausubel et al. (1987), supra, chapter 9; Sambrook et al. (2001), supra; and Campbell et al., Curr. Genet. 16: 53-56 (1989). The expression of heterologous protein in *Trichoderma* is described, for example, in U.S. Patent No. 6,022,725; U.S. Patent No. 6,268,328; Harkki et al.,
20 Enzyme Microb. Technol. 13: 227-233 (1991); Harkki et al., BioTechnol. 7: 596-603 (1989); EP 244,234; and EP 215,594. In one embodiment, genetically stable transformants are constructed with vector systems whereby the nucleic acid encoding a variant is stably integrated into a host cell chromosome. Transformants are then purified by known techniques.

25 In one non-limiting example, stable transformants including an *amdS* marker are distinguished from unstable transformants by their faster growth rate and the formation of circular colonies with a smooth, rather than ragged outline on solid culture medium containing acetamide. Additionally, in some cases a further test of stability is conducted by growing the transformants on solid non-selective medium, e.g., a medium that lacks
30 acetamide, harvesting spores from this culture medium and determining the percentage of these spores that subsequently germinate and grow on selective medium containing acetamide. Other methods known in the art may be used to select transformants.

3.3. Identification of activity

To evaluate the expression of a variant in a host cell, assays can measure the expressed protein, corresponding mRNA, or α -amylase activity. For example, suitable assays include Northern and Southern blotting, RT-PCR (reverse transcriptase polymerase chain reaction), and in situ hybridization, using an appropriately labeled hybridizing probe. Suitable assays also include measuring activity in a sample. Suitable assays of the exo-activity of the variant include, but are not limited to, the Betamyl® assay (Megazyme, Ireland). Suitable assays of the endo-activity of the variant include, but are not limited to, the Phadebas blue assay (Pharmacia and Upjohn Diagnostics AB). Assays also include HPLC analysis of liquefact prepared in the presence of the variant. HPLC can be used to measure amylase activity by separating DP-3 and DP-4 saccharides from other components of the assay.

Improved Handling Properties

The variant polypeptides described here preferably have improved properties when compared to SEQ ID NO:1, such as any one or more of improved thermostability, improved pH stability, or improved exo-specificity. The variant polypeptides described here preferably also have improved handling properties, such that a food product treated with a variant polypeptide has any one or all of lower firmness, higher resilience, higher cohesiveness, lower crumbliness, or higher foldability compared to a food product which has been treated with SEQ ID NO:1.

Without wishing to be bound by any particular theory, we believe that the mutations at the particular positions have individual and cumulative effects on the properties of a polypeptide comprising such mutations.

Thermostability and pH Stability

Preferably, the variant polypeptide is thermostable; preferably, it has higher thermostability than SEQ ID NO:1.

In wheat and other cereals the external side chains in amylopectin are in the range of DP 12-19. Thus, enzymatic hydrolysis of the amylopectin side chains, for example, by variant polypeptides as described having non-maltogenic exoamylase activity, can markedly reduce their crystallisation tendencies.

Starch in wheat and other cereals used for baking purposes is present in the form of starch granules which generally are resistant to enzymatic attack by amylases. Thus starch modification is mainly limited to damaged starch and is progressing very slowly during dough

processing and initial baking until gelatinisation starts at about 60°C. As a consequence hereof only amylases with a high degree of thermostability are able to modify starch efficiently during baking. And generally the efficiency of amylases is increased with increasing thermostability. That is because the more thermostable the enzyme is the longer time it can be active during baking and thus the more antistaling effect it will provide.

Accordingly, the use of variant polypeptides as described here when added to the starch at any stage of its processing into a food product, e.g., before during or after baking into bread can retard or impede or slow down the retrogradation. Such use is described in further detail below.

As used herein the term "thermostable" relates to the ability of the enzyme to retain activity after exposure to elevated temperatures. Preferably, the variant polypeptide is capable of degrading starch at temperatures of from about 55°C to about 80°C or more. Suitably, the enzyme retains its activity after exposure to temperatures of up to about 95°C.

The thermostability of an enzyme such as a non-maltogenic exoamylase is measured by its half life. Thus, the variant polypeptides described here have half lives extended relative to the parent enzyme by preferably 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or more, preferably at elevated temperatures of from 55°C to about 95°C or more, preferably at about 80°C.

As used here, the half life ($t_{1/2}$) is the time (in minutes) during which half the enzyme activity is inactivated under defined heat conditions. In preferred embodiments, the half life is assayed at 80 degrees C. Preferably; the sample is heated for 1-10 minutes at 80°C or higher. The half life value is then calculated by measuring the residual amylase activity, by any of the methods described here. Preferably, a half life assay is conducted as described in more detail in the Examples.

Preferably, the variant polypeptides described here are active during baking and hydrolyse starch during and after the gelatinization of the starch granules which starts at temperatures of about 55 degrees C. The more thermostable the non-maltogenic exoamylase is the longer time it can be active and thus the more antistaling effect it will provide. However, during baking above temperatures of about 85 degrees C, enzyme inactivation can take place. If this happens, the non-maltogenic exoamylase may be gradually inactivated so that there is substantially no activity after the baking process in the final bread. Therefore preferentially the non-maltogenic exoamylases suitable for use as described have an optimum temperature above 50 degrees C and below 98 degrees C.

The thermostability of the variant polypeptides described here can be improved by using protein engineering to become more thermostable and thus better suited for the uses described here; we therefore encompass the use of variant polypeptides modified to become more thermostable by protein engineering.

- 5 Preferably, the variant polypeptide is pH stable; more preferably, it has a higher pH stability than SEQ ID NO:1. As used herein the term "pH stable" relates to the ability of the enzyme to retain activity over a wide range of pHs. Preferably, the variant polypeptide is capable of degrading starch at a pH of from about 5 to about 10.5. In one embodiment, the degree of pH stability may be assayed by measuring the half life of the enzyme in specific pH
- 10 conditions. In another embodiment, the degree of pH stability may be assayed by measuring the activity or specific activity of the enzyme in specific pH conditions. The specific pH conditions may be any pH from pH5 to pH10.5.

Thus, the variant polypeptide may have a longer half life, or a higher activity (depending on the assay) when compared to SEQ ID NO:1 under identical conditions. The variant

15 polypeptides may have 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or longer half life when compared to SEQ ID NO:1 under identical pH conditions. Alternatively, or in addition, they may have such higher activity when compared to SEQ ID NO:1 under identical pH conditions.

Exo-Specificity

- 20 It is known that some non-maltogenic exoamylases can have some degree of endoamylase activity. In some cases, this type of activity may need to be reduced or eliminated since endoamylase activity can possibly negatively effect the quality of the final bread product by producing a sticky or gummy crumb due to the accumulation of branched dextrins.

Exo-specificity can usefully be measured by determining the ratio of total amylase activity to

25 the total endoamylase activity. This ratio is referred to in this document as a "Exo-specificity index". In preferred embodiments, an enzyme is considered an exoamylase if it has a exo-specificity index of 20 or more, i.e., its total amylase activity (including exo-amylase activity) is 20 times or more greater than its endoamylase activity. In highly preferred embodiments, the exo-specificity index of exoamylases is 30 or more, 40 or more, 50 or more, 60 or more,

30 70 or more, 80 or more, 90 or more, or 100 or more. In highly preferred embodiments, the exo-specificity index is 150 or more, 200 or more, 300 or more, 400 or more, 500 or more or 600 or more.

The total amylase activity and the endoamylase activity may be measured by any means known in the art. For example, the total amylase activity may be measured by assaying the total number of reducing ends released from a starch substrate. Alternatively, the use of a Betamyl assay is described in further detail in the Examples, and for convenience, amylase activity as assayed in the Examples is described in terms of "Betamyl Units" in the Tables.

Endoamylase activity may be assayed by use of a Phadebas Kit (Pharmacia and Upjohn). This makes use of a blue labelled crosslinked starch (labelled with an azo dye); only internal cuts in the starch molecule release label, while external cuts do not do so. Release of dye may be measured by spectrophotometry. Accordingly, the Phadebas Kit measures endoamylase activity, and for convenience, the results of such an assay are referred to in this document as "Phadebas units".

In a highly preferred embodiment, therefore, the exo-specificity index is expressed in terms of Betamyl Units / Phadebas Units, also referred to as "B/Phad".

Exo-specificity may also be assayed according to the methods described in the prior art, for example, in our International Patent Publication Number WO99/50399. This measures exo-specificity by way of a ratio between the endoamylase activity to the exoamylase activity. Thus, in a preferred aspect, the variant polypeptide described here will have less than 0.5 endoamylase units (EAU) per unit of exoamylase activity. Preferably the non-maltogenic exoamylases which are suitable for use according to the present invention have less than 0.05 EAU per unit of exoamylase activity and more preferably less than 0.01 EAU per unit of exoamylase activity.

The variant polypeptide described here will preferably have exospecificity, for example measured by exo-specificity indices, as described above, consistent with their being exoamylases. Moreover, they preferably have higher or increased exospecificity when compared to SEQ ID NO:1. Thus, for example, the variant polypeptide may have 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or higher exo-specificity index when compared to SEQ ID NO:1, preferably under identical conditions. They may have 1.5x or higher, 2x or higher, 5 x or higher, 10 x or higher, 50 x or higher, 100 x or higher, when compared to SEQ ID NO:1, preferably under identical conditions.

3.4. Methods for purifying variants

In general, a variant produced in cell culture is secreted into the medium and may be purified or isolated, e.g., by removing unwanted components from the cell culture medium. In some cases, a variant may be recovered from a cell lysate. In such cases, the enzyme is purified

from the cells in which it was produced using techniques routinely employed by those of skill in the art. Examples include, but are not limited to, affinity chromatography, ion-exchange chromatographic methods, including high resolution ion-exchange, hydrophobic interaction chromatography, two-phase partitioning, ethanol precipitation, reverse phase HPLC, chromatography on silica or on a cation-exchange resin, such as DEAE, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, and gel filtration using Sephadex G-75, for example.

4. Compositions and uses of variants

A polypeptide variant produced and purified by the methods described above is useful for a variety of industrial applications. In one embodiment, the polypeptide variant as defined herein is useful in a starch conversion process, particularly in a liquefaction process of a starch, e.g., corn starch, wheat starch, or barley starch. The desired end-product may be any product that may be produced by the enzymatic conversion of the starch substrate. For example, the desired product may be a syrup rich in saccharides useful for fermentation, particularly maltotriose, glucose, and/or maltose. The end product then can be used directly in a fermentation process to produce alcohol for fuel or drinking (i.e., potable alcohol). The skilled artisan is aware of various fermentation conditions that may be used in the production of ethanol or other fermentation end-products. A microbial organism capable of fermenting maltotrioses and/or less complex sugars, such as *S. cerevisiae* or a genetically modified variant thereof, is particularly useful. Suitable genetically altered variants of *S. cerevisiae* particularly useful for fermenting maltotrioses include variants that express AGT1 permease (Stambuck et al., Lett. Appl. Microbiol. 43: 370-76 (2006)), MTT1 and MTT1alt (Dietvorst et al., Yeast 22: 775-88 (2005)), or MAL32 (Dietvorst et al., Yeast 24: 27-38 (2007)). The present polypeptide variants also are useful in compositions and methods of food preparation, where enzymes that express amylase activity at high temperatures are desired.

The desirability of using a particular polypeptide variant will depend on the overall properties displayed by the polypeptide variant relative to the requirements of a particular application. As a general matter, polypeptide variants useful for a starch conversion process have substantial endo-amylase activity compared to wild-type PS4 or SEQ ID NO:1, and/or have a lower exo- to endo-amylase activity compared to wild-type PS4 or SEQ ID NO:1. Such polypeptide variants may be particularly useful in a liquefaction process, when used alone or combination with other amylase variants, where internal cleavage of complex branching saccharides lowers the viscosity of the substrate. Some polypeptide variants useful for liquefaction, however, are expected to have an endo-amylase activity comparable or even lower than wild-type PS4. Useful polypeptide variants include those with more or less exo-amylase activity than the wild-type PS4 or the SEQ ID NO: 1 polypeptide, depending on the

application. Compositions may include one or a combination of amylase variants, each of which may display a different set of properties.

4.1. Preparation of starch substrates

Those of skill in the art are well aware of available methods that may be used to prepare starch substrates for use in the processes disclosed herein. For example, a useful starch substrate may be obtained from tubers, roots, stems, legumes, cereals or whole grain. More specifically, the granular starch comes from plants that produce high amounts of starch. For example, granular starch may be obtained from corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana, or potatoes. Corn contains about 60-68% starch; barley contains about 55-65% starch; millet contains about 75-80% starch; wheat contains about 60-65% starch; and polished rice contains 70-72% starch. Specifically contemplated starch substrates are cornstarch, wheat starch, and barley starch. The starch from a grain may be ground or whole and includes corn solids, such as kernels, bran and/or cobs. The starch may be highly refined raw starch or feedstock from starch refinery processes. Various starches also are commercially available. For example, cornstarch is available from Cerestar, Sigma, and Katayama Chemical Industry Co. (Japan); wheat starch is available from Sigma; sweet potato starch is available from Wako Pure Chemical Industry Co. (Japan); and potato starch is available from Nakaari Chemical Pharmaceutical Co. (Japan).

Maltodextrins are useful as starch substrates in embodiments of the present invention. Maltodextrins comprise starch hydrolysis products having about 20 or fewer dextrose (glucose) units. Typical commercial maltodextrins contain mixtures of polysaccharides including from about three to about nineteen linked dextrose units. Maltodextrins are defined by the FDA as products having a dextrose equivalence (DE) of less than 20. They are generally recognized as safe (GRAS) food ingredients for human consumption. Dextrose equivalence (DE) is a measure of reducing power compared to a dextrose (glucose) standard of 100. The higher the DE, the greater the extent of starch depolymerization, resulting in a smaller average polymer (polysaccharide) size, and the greater the sweetness. A particularly useful maltodextrin is MALTRIN® M040 obtained from cornstarch, available from Grain Processing Corp. (Muscatine, Iowa): DE 4.0-7.0; bulk density 0.51 g/cc; measured water content 6.38% by weight.

The starch substrate can be a crude starch from milled whole grain, which contains non-starch fractions, e.g., germ residues and fibers. Milling may comprise either wet milling or dry milling. In wet milling, whole grain is soaked in water or dilute acid to separate the grain into its component parts, e.g., starch, protein, germ, oil, kernel fibers. Wet milling efficiently

separates the germ and meal (i.e., starch granules and protein) and is especially suitable for production of syrups. In dry milling, whole kernels are ground into a fine powder and processed without fractionating the grain into its component parts. Dry milled grain thus will comprise significant amounts of non-starch carbohydrate compounds, in addition to starch.

- 5 Most ethanol comes from dry milling. Alternatively, the starch to be processed may be a highly refined starch quality, for example, at least about 90%, at least 95%, at least 97%, or at least 99.5% pure.

4.2. Saccharification, gelatinization and liquefaction of starch

- 10 As used herein, the term "liquefaction" or "liquefy" means a process by which starch is converted to less viscous and shorter chain dextrins. This process involves gelatinization of starch simultaneously with or followed by the addition of a polypeptide variant as described herein. A thermostable polypeptide variant as described herein is preferably used for this application. Additional liquefaction-inducing enzymes optionally may be added.

- 15 In some embodiments, the starch or maltodextrin substrate prepared as described above is slurried with water. The starch or maltodextrin slurry may contain starch as a weight percent of dry solids of about 10-55%, about 20-45%, about 30-45%, about 30-40%, or optionally about 30-35%. The α -amylases, e.g., bacterial α -amylases, including *Bacillus* α -amylases, is usually supplied, for example, at about 1500 units per kg dry matter of starch, for example. To optimize α -amylase stability and activity, the pH of the slurry may be
20 adjusted to the optimal pH for the used polypeptide variant. Other α -amylases may be added and may require different optimal conditions. Bacterial α -amylase remaining in the slurry following liquefaction may be deactivated by lowering pH in a subsequent reaction step or by removing calcium from the slurry.

- 25 The slurry of starch plus the polypeptide variant may be pumped continuously through a jet cooker, which is steam heated from about 85°C to up to 105°C. Gelatinization occurs very rapidly under these conditions, and the enzymatic activity, combined with the significant shear forces, begins the hydrolysis of the starch substrate. The residence time in the jet cooker is very brief. The partly gelatinized starch may be passed into a series of holding tubes maintained at about 85-105°C and held for about 5 min. to complete the gelatinization
30 process. These tanks may contain baffles to discourage back mixing. As used herein, the term "secondary liquefaction" refers the liquefaction step subsequent to primary liquefaction, when the slurry is allowed to cool to room temperature. This cooling step can be about 30 minutes to about 180 minutes, e.g. about 90 minutes to 120 minutes.

A polypeptide variant as defined herein can be added to the liquefied starch obtained by the process above or to a maltodextrin slurry at about 0.01 to about 1.0 kg/MTDS. 1 kg/MTDS = 0.1 % by weight dissolved solids. In one embodiment, a polypeptide as defined herein can be added to a liquefied starch or maltodextrin slurry at a treatment level in a range from about 0.001% by weight to about 0.01% by weight based on dissolved solids. In a typical embodiment, a polypeptide as defined herein can be added to a liquefied starch or maltodextrin slurry at a treatment level in a range from about 0.0025% by weight to about 0.01% by weight based on dissolved solids. In one embodiment, the polypeptide as defined herein is immobilized, and the liquefied starch or maltodextrin substrate is passed over the immobilized polypeptide as defined herein and converted to product in a continuous reaction. In this embodiment, the polypeptide as defined herein may be immobilized with additional enzymes, such as a pullulanase.

The production of maltotetraose may further comprise contacting the liquefied starch or other source of maltodextrins with an isoamylase, a protease, a cellulase, a hemicellulase, a lipase, a cutinase, or any combination thereof.

Also provided is a method of making a saccharide (e.g., maltotetraose) syrup, comprising adding a polypeptide variant as defined herein or a composition comprising the variant to a starch liquefact and saccharifying the starch liquefact to form the saccharide syrup. The polypeptide variant as defined herein may be added to the starch liquefact in a range from about 0.001% by weight to about 0.1% by weight based on dissolved solids. In one embodiment, the variant is added to the starch liquefact in a range from about 0.0025% by weight to about 0.01% by weight based on dissolved solids. The units of concentration also are expressed herein as kg of polypeptide variant per metric ton of dry solids (MTDS), where 1 kg/MTDS = 0.1 % by weight dissolved solids. The liquefied starch solution may be a slurry of liquefied starch at about 20-35% w/w dry solids. The starch may be obtained from corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana, or potatoes. The starch liquefact may be saccharified at about 55°C to about 65°C such as at about 60°C to about 65°C. The starch liquefact may be saccharified at about pH 5.0 to about pH 7.0. A pullulanase, isoamylase, pullulanase, protease, cellulase, hemicellulase, lipase, cutinase, or any combination thereof, may be added with the polypeptide variant to the starch liquefact. In one embodiment, the saccharide syrup may be fermented to produce ethanol. The saccharide syrup produced by the method may comprise at least about 40%, about 45%, about 50%, about 55%, or about 60% by weight maltotetraose based on total saccharide content.

In another aspect a method of making a saccharide syrup, including adding a polypeptide variant as defined herein and an alpha-amylase to granular starch and hydrolyzing the

granular starch to form the saccharide syrup is provided. In one embodiment, the granular starch liquefact is produced by an alpha-amylase. In one embodiment, the granular starch liquefact is an acid produced liquefact. In one embodiment the polypeptide variant as defined herein is added to the granular starch in a range from about 0.001% by weight to about 0.1% by weight based on dissolved solids. In another embodiment the polypeptide variant as defined herein is added to the granular starch in a range from about 0.0025% by weight to about 0.01% by weight based on dissolved solids. The granular starch can be obtained from starch from corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana, or potatoes.

In a particular embodiment the granular starch is saccharified at 55°C to 65°C such as 60°C to 65°C. In another embodiment the granular starch is saccharified at pH 5.0 to pH 7.0. It is envisioned that the method can also include fermenting the saccharide syrup to produce ethanol.

In one embodiment the method includes a step of adding an enzyme having debranching activity to the granular starch. The enzyme having debranching activity can include but is not limited to an isoamylase, a pullulanase, an isopullulanase, a neopullulanase or any combination thereof. It is also envisioned that the method can optionally include a further step of adding a protease, a cellulase, a hemicellulase, a lipase, a cutinase, a pectate liase or any combination thereof to the granular starch.

In one embodiment the saccharide syrup includes at least 35% by weight maltotetraose based on total saccharide content. Alternatively, the saccharide syrup includes at least 45% by weight maltotetraose based on total saccharide content. In another embodiment the saccharide syrup includes at least 50% by weight maltotetraose based on total saccharide content. In a further embodiment the saccharide syrup includes from 45% by weight to 60% by weight maltotetraose based on total saccharide content.

It is envisioned that the polypeptide variant as defined herein of the method can be immobilized.

In another aspect a method is provided for making IMO, including adding a) a polypeptide variant as defined herein, b) an alpha-amylase, and c) a transglucosidase to starch in the form of a starch liquefact or granular starch and saccharifying the starch to form IMO. It is envisioned that the IMO can be formed at an IMO number of at least 30, at least 40 and/or at least 45. In one embodiment the starch is obtained from corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana, or potatoes.

In another aspect a method is provided for making IMO, including adding a) a polypeptide variant, b) an alpha-amylase, and c) a transglucosidase to starch in the form of a starch

liquefact or granular starch and saccharifying the starch to form IMO. Any of a number of transglucosidase enzymes (TG) can be use, for example, TRANSGLUCOSIDASE L-500® (Danisco US Inc., Genencor Division).

5 It is envisioned that the IMO can be formed at an IMO number of at least 30, at least 40 and/or at least 45. In one embodiment the starch is obtained from corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana, or potatoes.

4.3. Processes of fermentation

10 Yeast typically from *Saccharomyces* spp. is added to the mash and the fermentation is ongoing for 24-96 hours, such as typically 35-60 hours. The temperature is between about 26-34°C, typically at about 32°C, and the pH is from about pH 3-6, typically around about pH 4-5.

15 In one embodiment, a batch fermentation process is used in a closed system, where the composition of the medium is set at the beginning of the fermentation and is not altered during the fermentation. At the beginning of the fermentation, the medium is inoculated with the desired microbial organism(s). In this method, fermentation is permitted to occur without the addition of any components to the system. Typically, a batch fermentation qualifies as a "batch" with respect to the addition of the carbon source, and attempts are often made to control factors such as pH and oxygen concentration. The metabolite and biomass compositions of the batch system change constantly up to the time the fermentation is stopped. Within batch cultures, cells progress through a static lag phase to a high growth log phase and finally to a stationary phase, where growth rate is diminished or halted. If untreated, cells in the stationary phase eventually die. In general, cells in log phase are responsible for the bulk of production of product.

25 A suitable variation on the standard batch system is the "fed-batch fermentation" system. In this variation of a typical batch system, the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression likely inhibits the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. Measurement of the actual substrate concentration in fed-batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors, such as pH, dissolved oxygen and the partial pressure of waste gases, such as CO₂. Batch and fed-batch fermentations are common and well known in the art.

Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor, and an equal amount of conditioned medium is removed

simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density, where cells are primarily in log phase growth. Continuous fermentation allows for the modulation of one or more factors that affect cell growth and/or product concentration. For example, in one embodiment, a limiting nutrient, such as the carbon source or nitrogen source, is maintained at a fixed rate and all other parameters are allowed to moderate. In other systems, a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions. Thus, cell loss due to medium being drawn off should be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes, as well as techniques for maximizing the rate of product formation, are well known in the art of industrial microbiology.

Following the fermentation, the mash is distilled to extract the ethanol. The ethanol obtained according to the process of the disclosure may be used as, e.g., fuel ethanol; drinking ethanol, i.e., potable neutral spirits or industrial ethanol. Left over from the fermentation is the grain, which is typically used for animal feed, either in liquid form or dried. Further details on how to carry out liquefaction, saccharification, fermentation, distillation, and recovery of ethanol are well known to the skilled person. According to the process of the disclosure, the saccharification and fermentation may be carried out simultaneously or separately.

5. Compositions and methods for baking and food preparation

In one aspect, compositions, including food additives, food products, bakery products, improver compositions, feed products including animal feeds, etc comprising such altered variants as described herein, such as those which have non-maltogenic exoamylase activity, as well as methods of making and using such polypeptides and the compositions are provided.

As noted above, the variant polypeptides as described herein may comprise one or more improved handling properties, preferably improved baking properties. Thus, the variant polypeptides as described herein may provide that the food products so treated have one or more of (preferably all of) a lower firmness, a higher resilience, a higher cohesiveness, a lower crumbliness or a higher foldability. Such improved handling or baking properties exhibited by the variant polypeptides as described herein are described in further detail below. In one aspect, a variant, in which the half life ($t_{1/2}$), preferably at 60 degrees C, is increased by 15% or more, preferably 50% or more, most preferably 100% or more, compared to the amino acid sequence of SEQ ID NO: 1, is provided.

In one aspect, a variant as described herein, in which a food product treated with the variant has any one or more, preferably all of the following properties: (a) lower firmness; (b) higher resilience; (c) higher cohesiveness; (d) lower crumbliness; and (e) higher foldability compared to a food product which has been treated with the amino acid sequence of SEQ ID NO: 1, is provided.

In one aspect, a variant as described herein, in which the resilience, cohesiveness or foldability of the food product is independently increased by 15% or more, preferably 50% or more, most preferably 100% or more, compared to a food product which has been treated with the amino acid sequence of SEQ ID NO: 1, is provided.

In one aspect, a variant as described herein, in which each of resilience cohesiveness and foldability of a food product treated with the variant is increased compared to a food product which has been treated with the amino acid sequence of SEQ ID NO: 1, is provided.

In one aspect, a variant as described herein, in which the firmness or the crumbliness of the food product is independently decreased by 15% or more, preferably 50% or more, most preferably 100% or more, relative to a food product which has been treated with the amino acid sequence of SEQ ID NO: 1, is provided.

In one aspect, a variant as described herein, in which each of the firmness and crumbliness of a food product treated with the polypeptide is decreased compared to a food product which has been treated with the amino acid sequence of SEQ ID NO: 1, is provided.

In one aspect, a variant as described herein comprising a fragment of at least 20 residues of a polypeptide as set out in the claims, in which the polypeptide has non-maltogenic exoamylase activity, is provided.

In one aspect, a variant as described herein has non-maltogenic exoamylase activity. Such as activity may be determined by the methods described in US 6,667,065 which is incorporated by reference herein.

In one aspect, the treatment of food products, particularly doughs and bakery products with such polypeptides, and such that the food products exhibit the desired qualities set out above are provided.

In one aspect, a process for treating a starch comprising contacting the starch with a variant as described herein and allowing the polypeptide to generate from the starch one or more linear products, is provided. In one aspect, the use of a variant as described herein in

preparing a food or feed product, is provided. In one aspect, a process of preparing a food or feed product comprising admixing a variant as described herein with a food or feed ingredient, is provided. In one aspect, the use, or a process, in which the food product comprises a dough or a dough product, preferably a processed dough product, is provided. In one aspect, the use or process, in which the food product is a bakery product, is provided. In one aspect, a process for making a bakery product comprising: (a) providing a starch medium; (b) adding to the starch medium a variant as described herein; and (c) applying heat to the starch medium during or after step (b) to produce a bakery product, is provided. In one aspect, a food product, feed product, dough product or a bakery product obtained by a process as defined in the claims, is provided.

In one aspect, the use of a variant as described herein, in a dough product to retard or reduce staling, preferably detrimental retrogradation, of the dough product, is provided.

In one aspect, the use of a variant as described herein, in a dough product to improve any one or more of firmness, resilience, cohesiveness, crumbliness or foldability of the dough product, is provided.

In one aspect, a combination of a variant as described herein, together with any one or more of the following:

(a) maltogenic alpha-amylase also called glucan 1,4- α -maltohydrolase (EC 3.2.1.133) from *Bacillus stearothermophilus*, or a variant, homologue, or mutants thereof which have maltogenic alpha-amylase activity;

(b) a bakery xylanase (EC 3.2.1.8) from e.g. *Bacillus* sp., *Aspergillus* sp., *Thermomyces* sp. or *Trichoderma* sp.;

(c) α -amylase (EC 3.2.1.1) from *Bacillus amyloliquefaciens* or from *Aspergillus* sp. or a variant, homologue, or mutants thereof which have alpha-amylase activity; and

(d) a lipase such as glycolipase from *Fusarium heterosporum*, is provided.

The variants as described herein preferably comprise one or more improved handling properties compared to a parent polypeptide or a wild type polypeptide, such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1. The improved handling properties may in preferred embodiments comprise improved baking properties.

Thus, the variant as described herein are in one aspect such that a food product treated with the variant polypeptide has an improved handling or preferably baking property compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1. The handling or baking property may be selected from the group consisting of:

firmness, resilience, cohesiveness, crumbliness and foldability.

These handling properties may be tested by any means known in the art. For example, firmness, resilience and cohesiveness may be determined by analysing bread slices by Texture Profile Analysis using for example a Texture Analyser, as e.g. described in 11.

10 Firmness

The variants described here are in one aspect such that a food product treated with the variant polypeptide has lower firmness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1.

- 15 The firmness is in preferred embodiments inversely correlated with the softness of the food product; thus, a higher softness may reflect a lower firmness, and vice versa.

Firmness may be measured by the "Firmness Evaluation Protocol" set out in example 11.

- 20 In one aspect, the variants described herein are such that a food product treated with the variant polypeptide has a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or more lower firmness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1. A food product treated with the variant polypeptides disclosed herein may have a 1.1x, 1.5x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x or more lower firmness compared to a food product which has been treated with a parent
- 25 polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1.

Resilience

- 30 In one aspect, the variants described herein are such that a food product treated with the variant polypeptide has a higher resilience compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1.

Resilience may be measured by the "Resilience Evaluation Protocol" set out in 11.

Thus in one aspect, the variants described herein are such that a food product treated with the variant polypeptide has a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or more higher resilience compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1. A food product treated with the variant polypeptide may have a 1.1x, 1.5x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x or more higher resilience compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1.

Cohesiveness

In one aspect, the variants described herein are such that a food product treated with the variant polypeptide has higher cohesiveness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1.

Cohesiveness may be measured by the "Cohesiveness Evaluation Protocol" set out in 11.

Thus in one aspect, the variants described here are such that a food product treated with the variant polypeptide as disclosed herein has a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or more higher cohesiveness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1. A food product treated with the variant polypeptide may have a 1.1x, 1.5x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x or more higher cohesiveness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1.

Crumbliness

In one aspect, the variants described here are such that a food product treated with the variant polypeptide has lower crumbliness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1.

Crumbliness may be measured by the "Crumbliness Evaluation Protocol" set out in Example 13.

Thus in one aspect, the variants described here are such that a food product treated with the variant polypeptide has a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or more lower crumbliness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1. A food product treated with the variant polypeptide may have a 1.1x, 1.5x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x or more lower crumbliness compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1.

Foldability

In one aspect, the variants described here are such that a food product treated with the variant polypeptide has higher foldability compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1.

Foldability is preferably measured by the "Foldability Evaluation Protocol" set out in Example 14.

Thus, the variants described here are such that a food product treated with the variant polypeptide has a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or more higher foldability compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1. A food product treated with the variant polypeptide may have a 1.1x, 1.5x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x or more higher foldability compared to a food product which has been treated with a parent polypeptide or a wild type polypeptide such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1.

In one aspect, the use of the variant polypeptides described here in combination with a xylanase for improving foldability are provided.

Further, a method for preparing a food product, the method comprising: (a) obtaining a non-maltogenic exoamylase; (b) introducing a mutation at any one or more of the positions of the

non-maltogenic exoamylase as set out in this document; (c) admixing the resulting polypeptide with a food ingredient is provided.

The variant polypeptides may be used to enhance the volume of bakery products such as bread. While not wishing to be bound by any particular theory, we believe that this results from the reduction in viscosity of the dough during heating (such as baking) as a result of the amylase shortening amylose molecules. This enables the carbon dioxide generated by fermentation to increase the size of the bread with less hindrance.

Thus, food products comprising or treated with variant polypeptides are expanded in volume when compared to products which have not been so treated, or treated with parent polypeptides such as a polypeptide of SEQ ID NO:1 or SEQ ID NOs: 2, 4, 6, 8 or 10, preferably SEQ ID NO:1. In other words, the food products have a larger volume of air per volume of food product. Alternatively, or in addition, the food products treated with variant polypeptides as described herein have a lower density, or weight (or mass) per volume ratio. In particularly preferred embodiments, the variant polypeptides are used to enhance the volume of bread. Volume enhancement or expansion is beneficial because it reduces the gumminess or starchiness of foods. Light foods are preferred by consumers, and the customer experience is enhanced. In preferred embodiments, the use of variant polypeptides enhances the volume by 10%, 20%, 30% 40%, 50% or more.

The variant polypeptides and nucleic acids described here may be used as – or in the preparation of - a food. In particular, they may be added to a food, i.e., as a food additive. The term “food” is intended to include both prepared food, as well as an ingredient for a food, such as a flour. In a preferred aspect, the food is for human consumption. The food may be in the form of a solution or as a solid – depending on the use and/or the mode of application and/or the mode of administration.

The variant polypeptides and nucleic acids may be used as a food ingredient. As used herein the term “food ingredient” includes a formulation, which is or can be added to functional foods or foodstuffs and includes formulations which can be used at low levels in a wide variety of products that require, for example, acidifying or emulsifying. The food ingredient may be in the form of a solution or as a solid – depending on the use and/or the mode of application and/or the mode of administration.

The variant polypeptides and nucleic acids disclosed here may be – or may be added to - food supplements. The variant polypeptides and nucleic acids disclosed here may be – or may be added to - functional foods. As used herein, the term “functional food” means food which is capable of providing not only a nutritional effect and/or a taste satisfaction, but is

also capable of delivering a further beneficial effect to consumer. Although there is no legal definition of a functional food, most of the parties with an interest in this area agree that they are foods marketed as having specific health effects.

The variant polypeptides may also be used in the manufacture of a food product or a foodstuff. Typical foodstuffs include dairy products, meat products, poultry products, fish products and dough products. The dough product may be any processed dough product, including fried, deep fried, roasted, baked, steamed and boiled doughs, such as steamed bread and rice cakes. In highly preferred embodiments, the food product is a bakery product.

Preferably, the foodstuff is a bakery product. Typical bakery (baked) products include bread - such as loaves, rolls, buns, bagels, pizza bases etc. pastry, pretzels, tortillas, cakes, cookies, biscuits, crackers etc.

The food products preferably benefit from one or more of the improved handling or baking properties of the variant polypeptides described here. The improved handling or baking property may be selected from the group consisting of: improved firmness, improved resilience, improved cohesiveness, improved crumbliness and improved foldability.

Further in one aspect, a method of modifying a food additive comprising a non-maltogenic exoamylase, the method comprising introducing a mutation at any one or more of the positions of the non-maltogenic exoamylase as set out in this document is provided. The same method can be used to modify a food ingredient, or a food supplement, a food product, or a foodstuff.

In one aspect, the use of variant polypeptides that are capable of retarding the staling of starch media, such as starch gels are provided. The variant polypeptides are especially capable of retarding the detrimental retrogradation of starch.

Most starch granules are composed of a mixture of two polymers: an essentially linear amylose and a highly branched amylopectin. Amylopectin is a very large, branched molecule consisting of chains of α -D-glucopyranosyl units joined by (1-4) linkages, wherein said chains are attached by α -D-(1-6) linkages to form branches. Amylopectin is present in all natural starches, constituting about 75% of most common starches. Amylose is essentially a linear chain of (1-4) linked α -D-glucopyranosyl units having few α -D-(1-6) branches. Most starches contain about 25% amylose.

Starch granules heated in the presence of water undergo an order-disorder phase transition called gelatinization, where liquid is taken up by the swelling granules. Gelatinization

temperatures vary for different starches. Upon cooling of freshly baked bread the amylose fraction, within hours, retrogrades to develop a network. This process is beneficial in that it creates a desirable crumb structure with a low degree of firmness and improved slicing properties. More gradually crystallisation of amylopectin takes place within the gelatinised starch granules during the days after baking. In this process amylopectin is believed to reinforce the amylose network in which the starch granules are embedded. This reinforcement leads to increased firmness of the bread crumb. This reinforcement is one of the main causes of bread staling.

It is known that the quality of baked products gradually deteriorates during storage. As a consequence of starch recystallisation (also called retrogradation), the water-holding capacity of the crumb is changed with important implications on the organoleptic and dietary properties. The crumb loses softness and elasticity and becomes firm and crumbly. The increase in crumb firmness is often used as a measure of the staling process of bread.

The rate of detrimental retrogradation of amylopectin depends on the length of the side chains of amylopectin. Thus, enzymatic hydrolysis of the amylopectin side chains, for example, by variant polypeptides as described herein having non-maltogenic exoamylase activity, can markedly reduce their crystallisation tendencies.

Accordingly, in one aspect the use of variant polypeptides as described here when added to the starch at any stage of its processing into a food product e.g., before during or after baking into bread can retard or impede or slow down the retrogradation. Such use is described in further detail below.

In one aspect, a method of improving the ability of a non-maltogenic exoamylase to prevent staling, preferably detrimental retrogradation, of a dough product, the method comprising introducing a mutation at any one or more of the positions of the non-maltogenic exoamylase as set out in this document are provided.

For evaluation of the antistaling effect of the variant polypeptides such a variant having non-maltogenic exoamylase activity described here, the crumb firmness can be measured 1, 3 and 7 days after baking by means of an Instron 4301 Universal Food Texture Analyzer or similar equipment known in the art.

Another method used traditionally in the art and which is used to evaluate the effect on starch retrogradation of a variant polypeptide such as a variant having exoamylase activity is based on DSC (differential scanning calorimetry). Here, the melting enthalpy of retrograded amylopectin in bread crumb or crumb from a model system dough baked with or without

enzymes (control) is measured. The DSC equipment applied may be a Mettler-Toledo DSC 820 run with a temperature gradient of 10°C per min. from 20 to 95°C. For preparation of the samples 10-20 mg of crumb are weighed and transferred into Mettler-Toledo aluminium pans which then are hermetically sealed.

- 5 The model system doughs may contain standard wheat flour and optimal amounts of water or buffer with or without the polypeptide variant. They are mixed in a 10 or 50 g Brabender Farinograph for 6 or 7 min., respectively. Samples of the doughs are placed in glass test tubes (15* 0.8 cm) with a lid. These test tubes are subjected to a baking process in a water bath starting with 30 min. incubation at 33°C followed by heating from 33 to 95°C with a
10 gradient of 1.1°C per min. and finally a 5 min. incubation at 95°C. Subsequently, the tubes are stored in a thermostat at 20°C prior to DSC analysis.

- In one embodiment, the variants described here have a reduced melting enthalpy, compared to the control. In a further embodiment, the variants have a 10% or more reduced melting enthalpy. In yet a further aspect, they have a 20% or more, 30%, 40%, 50%, 60%, 70%,
15 80%, 90% or more reduced melting enthalpy when compared to the control.

- In one aspect, the use of variant polypeptides in the preparation of food products, in particular, starch products is provided. The method comprises forming the starch product by adding a non-maltogenic exoamylase enzyme such as a variant polypeptide, to a starch medium. If the starch medium is a dough, then the dough is prepared by mixing together
20 flour, water, the non-maltogenic exoamylase which is a variant polypeptide as described herein and optionally other possible ingredients and additives.

- The term "starch" should be taken to mean starch per se or a component thereof, especially amylopectin. The term "starch medium" means any suitable medium comprising starch. The term "starch product" means any product that contains or is based on or is derived from
25 starch. Preferably, the starch product contains or is based on or is derived from starch obtained from wheat flour. The term "flour" as used herein is a synonym for the finely-ground meal of wheat or other grain. Preferably, however, the term means flour obtained from wheat per se and not from another grain. Thus, and unless otherwise expressed, references to "wheat flour" as used herein preferably mean references to wheat flour per se
30 as well as to wheat flour when present in a medium, such as a dough.

A preferred flour is wheat flour or rye flour or mixtures of wheat and rye flour. However, dough comprising flour derived from other types of cereals such as for example from rice, maize, barley, and durra are also contemplated. Preferably, the starch product is a bakery product. More preferably, the starch product is a bread product. Even more preferably, the

starch product is a baked farinaceous bread product. The term "baked farinaceous bread product " refers to any baked product based on a dough obtainable by mixing flour, water, and a leavening agent under dough forming conditions. Further components can of course be added to the dough mixture.

5 Thus, if the starch product is a baked farinaceous bread product, then the process comprises mixing - in any suitable order - flour, water, and a leavening agent under dough forming conditions and further adding a variant polypeptide as described herein, optionally in the form of a premix. The leavening agent may be a chemical leavening agent such as sodium bicarbonate or any strain of *Saccharomyces cerevisiae* (Baker's Yeast).

10 The variant polypeptide as described herein can be added together with any dough ingredient including the water or dough ingredient mixture or with any additive or additive mixture. The dough can be prepared by any conventional dough preparation method common in the baking industry or in any other industry making flour dough based products.

Baking of farinaceous bread products such as for example white bread, bread made from
15 bolted rye flour and wheat flour, rolls and the like is typically accomplished by baking the bread dough at oven temperatures in the range of from 180 to 250°C for about 15 to 60 minutes. During the baking process a steep temperature gradient (200 to 120°C) is prevailing in the outer dough layers where the characteristic crust of the baked product is developed. However, owing to heat consumption due to steam generation, the temperature
20 in the crumb is only close to 100°C at the end of the baking process.

A process for making a bread product comprising: (a) providing a starch medium; (b) adding to the starch medium a variant polypeptide as described in this document; and (c) applying heat to the starch medium during or after step (b) to produce a bread product is provided. A
25 process for making a bread product comprising adding to a starch medium a variant polypeptide as described is also provided.

In one aspect, the variant polypeptide can be added as a liquid preparation or as a dry pulverulent composition either comprising the enzyme as the sole active component or in admixture with one or more additional dough ingredient or dough additive.

In a further aspect, the improver compositions, which include bread improving compositions
30 and dough improving compositions are provided. These comprise a variant polypeptide, optionally together with a further ingredient, or a further enzyme, or both.

In one aspect, an improver composition for a dough, in which the improver composition comprises a variant as set out in any of the claims, and at least one further dough ingredient or dough additive, is provided.

5 In one aspect, a composition comprising a flour and a variant as set out in any of the claims, is provided.

In a further aspect, the use of such a bread and dough improving compositions in baking is provided. In a further aspect, a baked product or dough obtained from the bread improving composition or dough improving composition is provided. In another aspect, a baked product or dough obtained from the use of a bread improving composition or a dough improving
10 composition is provided.

A dough may be prepared by admixing flour, water, a dough improving composition comprising variant polypeptide (as described above) and optionally other ingredients and additives.

15 The dough improving composition can be added together with any dough ingredient including the flour, water or optional other ingredients or additives. The dough improving composition can be added before the flour or water or optional other ingredients and additives. The dough improving composition can be added after the flour or water, or optional other ingredients and additives. The dough can be prepared by any conventional dough preparation method common in the baking industry or in any other industry making flour dough based products.

20 The dough improving composition can be added as a liquid preparation or in the form of a dry powder composition either comprising the composition as the sole active component or in admixture with one or more other dough ingredients or additive.

25 The amount of the variant polypeptide that is added is normally in an amount which results in the presence in the finished dough of 50 to 100,000 units per kg of flour, preferably 100 to 50,000 units per kg of flour. Preferably, the amount is in the range of 200 to 20,000 units per kg of flour. Alternatively, the variant polypeptide is added in an amount which results in the presence in the finished dough of 0.02 - 50 ppm of enzyme based on flour (0.02 - 50 mg enzyme per kg of flour), preferably 0.2 - 10 ppm.

30 In the present context, 1 unit of the non-maltogenic exoamylase is defined as the amount of enzyme which releases hydrolysis products equivalent to 1 μ mol of reducing sugar per min. when incubated at 50 degrees C in a test tube with 4 ml of 10 mg/ml waxy maize starch in 50 mM MES, 2 mM calcium chloride, pH 6.0 as described hereinafter.

The dough as described here generally comprises wheat meal or wheat flour and/or other types of meal, flour or starch such as corn flour, corn starch, maize flour, rice flour, rye meal, rye flour, oat flour, oat meal, soy flour, sorghum meal, sorghum flour, potato meal, potato flour or potato starch. The dough may be fresh, frozen, or part-baked.

- 5 The dough may be a leavened dough or a dough to be subjected to leavening. The dough may be leavened in various ways, such as by adding chemical leavening agents, e.g., sodium bicarbonate or by adding a leaven (fermenting dough), but it is preferred to leaven the dough by adding a suitable yeast culture, such as a culture of *Saccharomyces cerevisiae* (baker's yeast), e.g. a commercially available strain of *S. cerevisiae*.
- 10 The dough may comprise fat such as granulated fat or shortening. The dough may further comprise a further emulsifier such as mono- or diglycerides, sugar esters of fatty acids, polyglycerol esters of fatty acids, lactic acid esters of monoglycerides, acetic acid esters of monoglycerides, polyoxethylene stearates, or lysolecithin.

- A pre-mix comprising flour together with the combination as described herein are
- 15 furthermore provided. The pre-mix may contain other dough-improving and/or bread-improving additives, e.g. any of the additives, including enzymes, mentioned herein.

- In order to improve further the properties of the baked product and impart distinctive qualities to the baked product further dough ingredients and/or dough additives may be incorporated into the dough. Typically, such further added components may include dough
- 20 ingredients such as salt, grains, fats and oils, sugar or sweetener, dietary fibres, protein sources such as milk powder, gluten soy or eggs and dough additives such as emulsifiers, other enzymes, hydrocolloids, flavouring agents, oxidising agents, minerals and vitamins

- The emulsifiers are useful as dough strengtheners and crumb softeners. As dough
- 25 strengtheners, the emulsifiers can provide tolerance with regard to resting time and tolerance to shock during the proofing. Furthermore, dough strengtheners will improve the tolerance of a given dough to variations in the fermentation time. Most dough strengtheners also improve on the oven spring which means the increase in volume from the proofed to the baked goods. Lastly, dough strengtheners will emulsify any fats present in the recipe mixture.

- Suitable emulsifiers include lecithin, polyoxyethylene stearat, mono- and diglycerides of
- 30 edible fatty acids, acetic acid esters of mono- and diglycerides of edible fatty acids, lactic acid esters of mono- and diglycerides of edible fatty acids, citric acid esters of mono- and diglycerides of edible fatty acids, diacetyl tartaric acid esters of mono- and diglycerides of

edible fatty acids, sucrose esters of edible fatty acids, sodium stearyl-2-lactylate, and calcium stearyl-2-lactylate.

The further dough additive or ingredient can be added together with any dough ingredient including the flour, water or optional other ingredients or additives, or the dough improving composition. The further dough additive or ingredient can be added before the flour, water, optional other ingredients and additives or the dough improving composition. The further dough additive or ingredient can be added after the flour, water, optional other ingredients and additives or the dough improving composition.

The further dough additive or ingredient may conveniently be a liquid preparation. However, the further dough additive or ingredient may be conveniently in the form of a dry composition.

In one aspect the further dough additive or ingredient is at least 1% the weight of the flour component of dough. In a further aspect, the further dough additive or ingredient is at least 2%, preferably at least 3%, preferably at least 4%, preferably at least 5%, preferably at least 6%. If the additive is a fat, then typically the fat may be present in an amount of from 1 to 5%, typically 1 to 3%, more typically about 2%.

For the commercial and home use of flour for baking and food production, it is important to maintain an appropriate level of α -amylase activity in the flour. A level of activity that is too high may result in a product that is sticky and/or doughy and therefore unmarketable. Flour with insufficient α -amylase activity may not contain enough sugar for proper yeast function, resulting in dry, crumbly bread, or baked products. Accordingly, a variant as described herein, by itself or in combination with another α -amylase(s) or another variant, may be added to the flour to augment the level of endogenous α -amylase activity in flour. The variant in this embodiment can have a temperature optimum in the presence of starch in the ranges of about 30-90°C, 40-80°C, 40-50°C, 45-65°C, or 50-60°C, for example. The pH optimum in a 1% solution of soluble starch may be between pH 4.5 to 6.0, for example.

Grains, such as barley, oats, wheat, as well as plant components, such as corn, hops, and rice, also are used for brewing, both in industry and for home brewing. The components used in brewing may be unmalted or may be malted, i.e., partially germinated, resulting in an increase in the levels of enzymes, including α -amylase. For successful brewing, adequate levels of α -amylase enzyme activity are necessary to ensure the appropriate levels of sugars for fermentation. A variant, by itself or in combination with another α -amylase(s), accordingly may be added to the components used for brewing.

As used herein, the term "flour" means milled or ground cereal grain. The term "flour" also may mean Sago or tuber products that have been ground or mashed. In some embodiments, flour may also contain components in addition to the milled or mashed cereal or plant matter. An example of an additional component, although not intended to be limiting, is a leavening agent. Cereal grains include wheat, oat, rye, and barley. Tuber products include tapioca flour, cassava flour, and custard powder. The term "flour" also includes ground corn flour, maize-meal, rice flour, whole-meal flour, self-rising flour, tapioca flour, cassava flour, ground rice, enriched flower, and custard powder.

As used herein, the term "stock" means grains and plant components that are crushed or broken. For example, barley used in beer production is a grain that has been coarsely ground or crushed to yield a consistency appropriate for producing a mash for fermentation. As used herein, the term "stock" includes any of the aforementioned types of plants and grains in crushed or coarsely ground forms. The methods described herein may be used to determine α -amylase activity levels in both flours and stock.

One or more further enzymes may be used in combination with the variant polypeptides as described herein. Such combinations may for example be added to the food, dough preparation, foodstuff or starch composition.

Baking amylases may be from a fungus, bacterium or plant. It may be an α -amylase (EC 3.2.1.1). The α -amylase may be a fungal α -amylase from *Aspergillus* or *Trichoderma*, e.g. from *Aspergillus oryzae*. Or it may be an α -amylase from *Bacillus*, e.g. *B. amyloliquefaciens* or *B. licheniformis*. Or it may be a plant β -amylase (EC 3.2.1.2), e.g. β -amylase from barley malt or soy bean. Or it may be an exo-amylase used for anti-staling, e.g. non-maltogenic exo-amylase (EC 3.2.1.60) developed from *Pseudomonas saccharophila* maltotetrahydrolase (SEQ ID NO: 10) or maltogenic amylase (EC 3.2.1.133) developed from *Bacillus stearothermophilus* as further described below.

In one aspect, the further amylase is a maltogenic α -amylase. A "maltogenic α -amylase" (glucan 1,4- α -maltohydrolase, E.C. 3.2.1.133) is able to hydrolyze amylose and amylopectin to maltose in the α -configuration. A maltogenic α -amylase from *Bacillus* (EP 120 693) is commercially available under the trade name Novamyl (Novozymes, Denmark) and is widely used in the baking industry as an anti-staling agent due to its ability to reduce retrogradation of starch. Novamyl is described in detail in International Patent Publication WO 91/04669. The maltogenic α -amylase Novamyl shares several characteristics with cyclodextrin glucanotransferases (CGTases), including sequence homology (Henrissat B, Bairoch A; *Biochem. J.*, 316, 695-696 (1996)) and formation of transglycosylation products (Christophersen, C., et al., 1997, *Starch*, vol. 50, No. 1, 39-45).

The Novamyl may in particular comprise Novamyl 1500 MG. Other documents describing Novamyl and its uses include Christophersen, C., Pedersen, S., and Christensen, T., (1993) Method for production of maltose and a limit dextrin, the limit dextrin, and use of the limit dextrin. Denmark, and WO 95/10627. It is further described in U.S. Pat. No. 4,598,048 and U.S. Pat. No. 4,604,355. Preferred examples of antistaling amylases are exo-amylases, e.g. non-maltogenic exo-amylase or G4-amylase (EC 3.2.1.60) developed from *Pseudomonas saccharophila* maltotetraohydrolase having SEQ ID NO: 1 or maltogenic amylase (EC 3.2.1.133) developed from *Bacillus stearothermophilus* having SEQ ID NO: 51.

An antistaling amylase reduces staling after baking and leads to a reduction in firmness increase and a reduction in resilience decrease from day 1 to day 7 after baking relative to a control without enzyme. An antistaling amylase can be assayed by doing a baking trial and using texture analysis to determine firmness and resilience development over time. Texture analysis is described in example 11.

The further enzymes may be selected from, for example, any combination of the following:

(a) Novamyl, or a variant, homologue, or mutants thereof which have maltogenic alpha-amylase activity; (b) a xylanase such as GRINDAMYL™ POWERBake 900 (Danisco A/S); (c) a bacterial α -amylase such as Max-Life U4 (Danisco A/S); and (d) a lipase such as GRINDAMYL™ POWERBake 4050 (Danisco A/S).

In one embodiment a variant polypeptide as described herein is used in combination with at least one enzyme selected from the list consisting of oxidoreductases, hydrolases, lipases, esterases, glycosidases, amylases, pullulanases, xylanases, cellulases, hemicellulases, starch degrading enzymes, proteases and lipoxygenases. In one embodiment, the composition comprises at least one variant polypeptide as described herein and a maltogenic amylase from *Bacillus*, as disclosed in WO91/04669. One embodiment comprises a variant polypeptide as described herein and flour.

Further enzymes that may be added to the dough include oxidoreductases, hydrolases, such as lipases and esterases as well as glycosidases like α -amylase, pullulanase, and xylanase. Oxidoreductases, such as for example glucose oxidase and hexose oxidase, can be used for dough strengthening and control of volume of the baked products and xylanases and other hemicellulases may be added to improve dough handling properties, crumb firmness and bread volume. Lipases are useful as dough strengtheners and crumb softeners and α -amylases and other amylolytic enzymes may be incorporated into the dough to control bread volume and further reduce crumb firmness.

Further enzymes that may be used may be selected from the group consisting of a cellulase, a hemicellulase, a starch degrading enzyme, a protease, a lipoxxygenase.

A variant as defined herein further can be added alone or in a combination with other amylases, including other amylase variants, to prevent or retard staling, i.e., crumb firming of baked products. The amount of anti-staling amylase will typically be in the range of 0.01-10 mg of enzyme protein per kg of flour, e.g., 0.5 mg/kg ds. Additional anti-staling amylases that can be used in combination with a variant polypeptide as described herein include an endo-amylase, e.g., a bacterial endo-amylase from *Bacillus*. The additional amylase can be a maltogenic α -amylase (EC 3.2.1.133), e.g., from *Bacillus*. Novamyl® is an exemplary maltogenic α -amylase from *B. stearothermophilus* strain NCIB 11837 and is described in Christophersen et al., *Starch* 50: 39-45 (1997). Other examples of anti-staling endo-amylases include bacterial α -amylases derived from *Bacillus*, such as *B. licheniformis* or *B. amyloliquefaciens*. The anti-staling amylase may be an exo-amylase, such as α -amylase, e.g., from plant sources, such as soybean, or from microbial sources, such as *Bacillus*.

The baking composition comprising a variant polypeptide as described herein further can comprise a phospholipase. The phospholipase may have A_1 or A_2 activity to remove fatty acid from the phospholipids, forming a lysophospholipid. It may or may not have lipase activity, i.e., activity on triglyceride substrates. The phospholipase typically has a temperature optimum in the range of 30-90°C, e.g., 30-70°C. The added phospholipases can be of animal origin, for example, from pancreas, e.g., bovine or porcine pancreas, snake venom or bee venom. Alternatively, the phospholipase may be of microbial origin, e.g., from filamentous fungi, yeast or bacteria, such as the genus or species. Exemplary sources of phospholipases include *Aspergillus*, *A. niger*; *Dictyostelium*, *D. discoideum*; *Mucor*, *M. javanicus*, *M. mucedo*, *M. subtilissimus*; *Neurospora*, *N. crassa*; *Rhizomucor*, *R. pusillus*; *Rhizopus*, *R. arrhizus*, *R. japonicus*, *R. stolonifer*; *Sclerotinia*, *S. libertiana*; *Trichophyton*, *T. rubrum*; *Whetzelinia*, *W. sclerotiorum*; *Bacillus*, *B. megaterium*, *B. subtilis*; *Citrobacter*, *C. freundii*; *Enterobacter*, *E. aerogenes*, *E. cloacae*; *Edwardsiella*, *E. tarda*; *Ectwinia*, *E. herbicola*; *Escherichia*, *E. coli*; *Klebsiella*, *K. pneumoniae*; *Proteus*, *P. vulgaris*; *Providencia*, *P. stuartii*; *Salmonella*, *S. typhimurium*; *Serratia*, *S. liquefaciens*, *S. marcescens*; *Shigella*, *S. flexneri*; *Streptomyces*, *S. violaceoruber*; *Yersinia*, *Y. enterocolitica*; *Fusarium*, and *F. oxysporum* (strain DSM 2672, for example).

The phospholipase is added in an amount that improves the softness of the bread during the initial period after baking, particularly the first 24 hours. The amount of phospholipase will typically be in the range of about 0.01-10 mg of enzyme protein per kg of flour, e.g., 0.1-5 mg/kg. Phospholipase activity generally will be in the range of about 20-1000 Lipase Unit (LU)/kg of flour, where a Lipase Unit is defined as the amount of enzyme required to release

1 μ mol butyric acid per minute at 30 °C, pH 7.0, with gum arabic as emulsifier and tributyrin as substrate.

Compositions of dough generally comprise wheat meal or wheat flour and/or other types of meal, flour or starch such as corn flour, cornstarch, rye meal, rye flour, oat flour, oatmeal,
5 soy flour, sorghum meal, sorghum flour, potato meal, potato flour or potato starch. The dough may be fresh, frozen or par-baked. The dough can be a leavened dough or a dough to be subjected to leavening. The dough may be leavened in various ways, such as by adding chemical leavening agents, e.g., sodium bicarbonate or by adding a leaven, i.e., fermenting dough. Dough also may be leavened by adding a suitable yeast culture, such as a culture of
10 *Saccharomyces cerevisiae* (baker's yeast), e.g., a commercially available strain of *S. cerevisiae*.

The dough may also comprise other conventional dough ingredients, e.g., proteins, such as milk powder, gluten, and soy; eggs (e.g., whole eggs, egg yolks or egg whites); an oxidant, such as ascorbic acid, potassium bromate, potassium iodate, azodicarbonamide (ADA) or
15 ammonium persulfate; an amino acid such as L-cysteine; a sugar; or a salt, such as sodium chloride, calcium acetate, sodium sulfate or calcium sulfate. The dough further may comprise fat, e.g., triglyceride, such as granulated fat or shortening. The dough further may comprise an emulsifier such as mono- or diglycerides, diacetyl tartaric acid esters of mono- or diglycerides, sugar esters of fatty acids, polyglycerol esters of fatty acids, lactic acid esters
20 of monoglycerides, acetic acid esters of monoglycerides, polyoxyethyllylene stearates, or lysolecithin. In particular, the dough can be made without addition of emulsifiers.

Optionally, an additional enzyme may be used together with the anti-staling amylase and the phospholipase. The additional enzyme may be a second amylase, such as an amyloglucosidase, a α -amylase, a cyclodextrin glucanotransferase, or the additional enzyme
25 may be a peptidase, in particular an exopeptidase, a transglutaminase, a lipase, a cellulase, a hemicellulase, in particular a pentosanase, such as xylanase, a protease, a protein disulfide isomerase, e.g., a protein disulfide isomerase as disclosed in WO 95/00636, for example, a glycosyltransferase, a branching enzyme (1,4- α -glucan branching enzyme), a 4- α -glucanotransferase (dextrin glycosyltransferase) or an oxidoreductase, e.g., a peroxidase, a
30 laccase, a glucose oxidase, a pyranose oxidase, a lipooxygenase, an L-amino acid oxidase or a carbohydrate oxidase. The additional enzyme(s) may be of any origin, including mammalian and plant, and particularly of microbial (bacterial, yeast or fungal) origin and may be obtained by techniques conventionally used in the art.

The xylanase is typically of microbial origin, e.g., derived from a bacterium or fungus, such
35 as a strain of *Aspergillus*, in particular of *A. aculeatus*, *A. niger* (cf. WO 91/19782), *A.*

awamori (e.g., WO 91/18977), or *A. tubigensis* (e.g., WO 92/01793); from a strain of *Trichoderma*, e.g., *T. reesei*, or from a strain of *Humicola*, e.g., *H. insolens* (e.g., WO 92/17573). Pentopan® and Novozym 384® are commercially available xylanase preparations produced from *Trichoderma reesei*. The amyloglucosidase may be an *A. niger* amyloglucosidase (such as AMG®). Other useful amylase products include Grindamyl® A 1000 or A 5000 (available from Danisco, Denmark) and Amylase® H or Amylase® P (available from Gist-Brocades, The Netherlands). The glucose oxidase may be a fungal glucose oxidase, in particular an *Aspergillus niger* glucose oxidase (such as Gluzyme®). An exemplary protease is Neutrase®. An exemplary lipase can be derived from strains of *Thermomyces* (*Humicola*), *Rhizomucor*, *Candida*, *Aspergillus*, *Rhizopus*, or *Pseudomonas*, in particular from *Thermomyces lanuginosus* (*Humicola lanuginosa*), *Rhizomucor miehei*, *Candida antarctica*, *Aspergillus niger*, *Rhizopus delemar* or *Rhizopus arrhizus*, or *Pseudomonas cepacia*. In specific embodiments, the lipase may be Lipase A or Lipase B derived from *Candida antarctica* as described in WO 88/02775, for example, or the lipase may be derived from *Rhizomucor miehei* as described in EP 238,023, for example, or *Humicola lanuginosa*, described in EP 305,216, for example, or *Pseudomonas cepacia* as described in EP 214,761 and WO 89/01032, for example.

The process may be used for any kind of baked product prepared from dough, either of a soft or a crisp character, either of a white, light or dark type. Examples are bread, particularly white, whole-meal or rye bread, typically in the form of loaves or rolls, such as, but not limited to, French baguette-type bread, pita bread, tortillas, cakes, pancakes, biscuits, cookies, pie crusts, crisp bread, steamed bread, pizza and the like.

In another embodiment, a variant polypeptide as described herein may be used in a pre-mix, comprising flour together with an anti-staling amylase, a phospholipase and a phospholipid. The pre-mix may contain other dough-improving and/or bread-improving additives, e.g., any of the additives, including enzymes, mentioned above. In one aspect, the variant polypeptide as described herein is a component of an enzyme preparation comprising an anti-staling amylase and a phospholipase, for use as a baking additive.

The enzyme preparation is optionally in the form of a granulate or agglomerated powder. The preparation can have a narrow particle size distribution with more than 95% (by weight) of the particles in the range from 25 to 500 µm. Granulates and agglomerated powders may be prepared by conventional methods, e.g., by spraying the variant polypeptide as described herein onto a carrier in a fluid-bed granulator. The carrier may consist of particulate cores having a suitable particle size. The carrier may be soluble or insoluble, e.g., a salt (such as NaCl or sodium sulfate), a sugar (such as sucrose or lactose), a sugar alcohol (such as sorbitol), starch, rice, corn grits, or soy.

Another aspect contemplates the enveloping of particles comprising a variant polypeptide as described herein, i.e., $\bullet$ -amylase particles. To prepare the enveloped $\bullet$ -amylase particles, the enzyme is contacted with a food grade lipid in sufficient quantity to suspend all of the $\bullet$ -amylase particles. Food grade lipids, as used herein, may be any naturally organic compound that is insoluble in water but is soluble in non-polar organic solvents such as hydrocarbon or diethyl ether. Suitable food grade lipids include, but are not limited to, triglycerides either in the form of fats or oils that are either saturated or unsaturated. Examples of fatty acids and combinations thereof which make up the saturated triglycerides include, but are not limited to, butyric (derived from milk fat), palmitic (derived from animal and plant fat), and/or stearic (derived from animal and plant fat). Examples of fatty acids and combinations thereof which make up the unsaturated triglycerides include, but are not limited to, palmitoleic (derived from animal and plant fat), oleic (derived from animal and plant fat), linoleic (derived from plant oils), and/or linolenic (derived from linseed oil). Other suitable food grade lipids include, but are not limited to, monoglycerides and diglycerides derived from the triglycerides discussed above, phospholipids and glycolipids.

The food grade lipid, particularly in the liquid form, is contacted with a powdered form of the $\bullet$ -amylase particles in such a fashion that the lipid material covers at least a portion of the surface of at least a majority, e.g., 100% of the $\bullet$ -amylase particles. Thus, each $\bullet$ -amylase particle is individually enveloped in a lipid. For example, all or substantially all of the $\bullet$ -amylase particles are provided with a thin, continuous, enveloping film of lipid. This can be accomplished by first pouring a quantity of lipid into a container, and then slurring the $\bullet$ -amylase particles so that the lipid thoroughly wets the surface of each $\bullet$ -amylase particle. After a short period of stirring, the enveloped $\bullet$ -amylase particles, carrying a substantial amount of the lipids on their surfaces, are recovered. The thickness of the coating so applied to the particles of $\bullet$ -amylase can be controlled by selection of the type of lipid used and by repeating the operation in order to build up a thicker film, when desired.

The storing, handling and incorporation of the loaded delivery vehicle can be accomplished by means of a packaged mix. The packaged mix can comprise the enveloped $\bullet$ -amylase. However, the packaged mix may further contain additional ingredients as required by the manufacturer or baker. After the enveloped $\bullet$ -amylase has been incorporated into the dough, the baker continues through the normal production process for that product.

The advantages of enveloping the $\bullet$ -amylase particles are two-fold. First, the food grade lipid protects the enzyme from thermal denaturation during the baking process for those enzymes that are heat labile. Consequently, while the $\bullet$ -amylase is stabilized and protected during the proving and baking stages, it is released from the protective coating in the final baked good product, where it hydrolyzes the glucosidic linkages in polyglucans. The loaded

delivery vehicle also provides a sustained release of the active enzyme into the baked good. That is, following the baking process, active α -amylase is continually released from the protective coating at a rate that counteracts, and therefore reduces the rate of, staling mechanisms.

- 5 In general, the amount of lipid applied to the α -amylase particles can vary from a few percent of the total weight of the α -amylase to many times that weight, depending upon the nature of the lipid, the manner in which it is applied to the α -amylase particles, the composition of the dough mixture to be treated, and the severity of the dough-mixing operation involved.
- 10 The loaded delivery vehicle, i.e., the lipid-enveloped enzyme, is added to the ingredients used to prepare a baked good in an effective amount to extend the shelf-life of the baked good. The baker computes the amount of enveloped α -amylase, prepared as discussed above, that will be required to achieve the desired anti-staling effect. The amount of the enveloped α -amylase required is calculated based on the concentration of enzyme enveloped
- 15 and on the proportion of α -amylase to flour specified. A wide range of concentrations has been found to be effective, although, as has been discussed, observable improvements in anti-staling do not correspond linearly with the α -amylase concentration, but above certain minimal levels, large increases in α -amylase concentration produce little additional improvement. The α -amylase concentration actually used in a particular bakery production
- 20 could be much higher than the minimum necessary in order to provide the baker with some insurance against inadvertent under-measurement errors by the baker. The lower limit of enzyme concentration is determined by the minimum anti-staling effect the baker wishes to achieve.

A method of preparing a baked good may comprise: (a) preparing lipid-coated α -amylase particles, where substantially all of the α -amylase particles are coated; (b) mixing a dough containing flour; (c) adding the lipid-coated α -amylase to the dough before the mixing is complete and terminating the mixing before the lipid coating is removed from the α -amylase; (d) proofing the dough; and (e) baking the dough to provide the baked good, where the α -amylase is inactive during the mixing, proofing and baking stages and is active in the baked

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30 good.

The enveloped α -amylase can be added to the dough during the mix cycle, e.g., near the end of the mix cycle. The enveloped α -amylase is added at a point in the mixing stage that allows sufficient distribution of the enveloped α -amylase throughout the dough; however, the mixing stage is terminated before the protective coating becomes stripped from the α -amylase particle(s). Depending on the type and volume of dough, and mixer action and

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speed, anywhere from one to six minutes or more might be required to mix the enveloped •-amylase into the dough, but two to four minutes is average. Thus, several variables may determine the precise procedure. First, the quantity of enveloped •-amylase should have a total volume sufficient to allow the enveloped •-amylase to be spread throughout the dough mix. If the preparation of enveloped •-amylase is highly concentrated, additional oil may need to be added to the pre-mix before the enveloped •-amylase is added to the dough. Recipes and production processes may require specific modifications; however, good results generally can be achieved when 25% of the oil specified in a bread dough formula is held out of the dough and is used as a carrier for a concentrated enveloped •-amylase when added near the end of the mix cycle. In bread or other baked goods, particularly those having a low fat content, e.g., French-style breads, an enveloped •-amylase mixture of approximately 1% of the dry flour weight is sufficient to admix the enveloped •-amylase properly with the dough. The range of suitable percentages is wide and depends on the formula, finished product, and production methodology requirements of the individual baker. Second, the enveloped •-amylase suspension should be added to the mix with sufficient time for complete mixture into the dough, but not for such a time that excessive mechanical action strips the protective lipid coating from the enveloped •-amylase particles.

6. Textile desizing compositions and use

Also contemplated are compositions and methods of treating fabrics (e.g., to desize a textile) using a variant polypeptide as described herein. In one aspect, a method of desizing textiles, comprising contacting the variant polypeptide as described herein with a textile for a time sufficient to desize the textile, is provided.

Fabric-treating methods are well known in the art (see, e.g., U.S. Patent No. 6,077,316). For example, in one aspect, the feel and appearance of a fabric is improved by a method comprising contacting the fabric with a variant polypeptide as described herein in a solution. In one aspect, the fabric is treated with the solution under pressure.

In one aspect, a variant polypeptide as described herein is applied during or after the weaving of a textile, or during the desizing stage, or one or more additional fabric processing steps. During the weaving of textiles, the threads are exposed to considerable mechanical strain. Prior to weaving on mechanical looms, warp yarns are often coated with sizing starch or starch derivatives to increase their tensile strength and to prevent breaking. A variant polypeptide as described herein can be applied during or after the weaving to remove these sizing starch or starch derivatives. After weaving, a variant polypeptide as described herein can be used to remove the size coating before further processing the fabric to ensure a homogeneous and wash-proof result.

A variant polypeptide as described herein can be used alone or with other desizing chemical reagents and/or desizing enzymes to desize fabrics, including cotton-containing fabrics, as detergent additives, e.g., in aqueous compositions. A variant polypeptide as described herein also can be used in compositions and methods for producing a stonewashed look on indigo-dyed denim fabric and garments. For the manufacture of clothes, the fabric can be cut and sewn into clothes or garments, which are afterwards finished. In particular, for the manufacture of denim jeans, different enzymatic finishing methods have been developed. The finishing of denim garment normally is initiated with an enzymatic desizing step, during which garments are subjected to the action of amylolytic enzymes to provide softness to the fabric and make the cotton more accessible to the subsequent enzymatic finishing steps. A variant polypeptide as disclosed herein can be used in methods of finishing denim garments (e.g., a "bio-stoning process"), enzymatic desizing and providing softness to fabrics, and/or finishing process.

Further embodiments of the invention

1. A polypeptide having amylase activity comprising an amino acid sequence having
 - a. at least 65 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 88 or 205, and/or
 - b. at least 78 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 16, 48, 97, 105, 235, 240, 248, 266, 311, 347, 350, 362, 364, 369, 393, 395, 396, 400, 401, 403, 412 or 409, and/or
 - c. at least 78 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more of the following amino acid substitutions: 42K/A/V/N/I/H/F, 34Q, 100Q/K/N/R, 272D, 392 K/D/E/Y/N/Q/R/T/G or 399C/H, and/or
 - d. at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 44, 96, 204, 354 or 377, and/or
 - e. at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises the following amino acid substitution: 392S

wherein an amino acid substitution is relative to the corresponding amino acid of SEQ ID NO: 1 and the positions are with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

2. A polypeptide having amylase activity comprising an amino acid sequence having at least 65 % sequence identity to the amino acid sequence of SEQ ID NO: 1, and wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 88, or 205 and/or one or more of the following amino acid substitutions: 42K, 235H/K/R, 240E, 392 K/D/E/Y or 409E wherein an amino acid substitution is relative to the corresponding amino acid of SEQ ID NO: 1 and the positions are with reference to the position numbering of the sequence shown as SEQ ID NO: 1.
3. The polypeptide according to embodiment 1, wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 88, 205, 235, 240, 248, 266, 311, 377 or 409 and/or one or more of the following amino acid substitutions: 42K/A/V/N/I/H/F, 34Q, 100Q/K/N/R, 272D or 392K/D/E/Y/N/Q/R/S/T/G.
4. The polypeptide according to any one of embodiments 1 and 3, wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 88, 205, 235, 240, 311 or 409 and/or one or more of the following amino acid substitutions: 42K/N/I/H/F, 272D, or 392 K/D/E/Y/N/Q/R/S/T/G.
5. The polypeptide according to any one of embodiments 1 and 3-4, wherein the polypeptide comprises amino acid substitutions at least in four, five or in all of the following positions: 88, 205, 235, 240, 311 or 409 and/or has at least one, or two the following amino acid substitutions: 42K/N/I/H/F, 272D or 392 K/D/E/Y/N/Q/R/S/T/G.
6. The polypeptide according to any one of embodiments 1-5, wherein the polypeptide further comprises one or more of the following amino acids 33Y, 34N, 70D, 121F, 134R, 141P, 146G, 157L, 161A, 178F, 179T, 223E/S/K/A, 229P, 307K, 309P or 334P.
7. The polypeptide according to any one of embodiments 1-6, wherein the polypeptide further has the following amino acids 33Y, 34N, 70D, 121F, 134R, 141P, 146G, 157L, 161A, 178F, 179T, 229P, 307K, 309P and 334P.
8. The polypeptide according to any one of embodiments 1-6, wherein the polypeptide has at least one of the amino acid substitutions in b) of embodiment 1 and further

comprises one or more amino acid substitutions at the following positions: 44, 96, 204, 354 or 377.

9. The polypeptide according to any one of embodiments 1-8 having at least 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 1.
10. The polypeptide according to any one of the embodiments 1-9, wherein the polypeptide has the amino acid 34Q.
11. The polypeptide according to any one of the embodiments 1 and 2-10, wherein the polypeptide has the amino acid 42K/F/H/I/N/A/V.
12. The polypeptide according to any one of the embodiments 1 and 2-10, wherein the polypeptide has the amino acid 42K/F/H/I/N.
13. The polypeptide according to any one of the embodiments 1-12, wherein the polypeptide has the amino acid 42K.
14. The polypeptide according to any one of the embodiments 1-13, wherein the polypeptide comprises an amino acid substitution in position 88.
15. The polypeptide according to any one of the embodiments 1-14, wherein the polypeptide has the amino acid 88L/Y/K.
16. The polypeptide according to any one of the embodiments 1-15, wherein the polypeptide has the amino acid 88L.
17. The polypeptide according to any one of the embodiments 1-16, wherein the polypeptide has the amino acid 88Y.
18. The polypeptide according to any one of the embodiments 1-17, wherein the polypeptide comprises an amino acid substitution in position 205.
19. The polypeptide according to any one of the embodiments 1-18, wherein the polypeptide has the amino acid 205L/K/M/N/Q/R/V/Y.

20. The polypeptide according to any one of the embodiments 1-19, wherein the polypeptide has the amino acid 205L/ K/M/N/Q/R/V.
21. The polypeptide according to any one of the embodiments 1-20, wherein the polypeptide has the amino acid 205L.
- 5 22. The polypeptide according to any one of the embodiments 1 and 2-21, wherein the polypeptide comprises an amino acid substitution in position 235.
23. The polypeptide according to any one of the embodiments 1 and 2-22, wherein the polypeptide has the amino acid 235H/ K/ R/ Q/ S.
- 10 24. The polypeptide according to any one of the embodiments 1 and 2-23, wherein the polypeptide has the amino acid 235H/ K/ R/ Q.
25. The polypeptide according embodiment 2 or 24, wherein the polypeptide has the amino acid 235H/ K/ R.
26. The polypeptide according to embodiment 2 or 25, wherein the polypeptide has the amino acid 235R.
- 15 27. The polypeptide according to embodiment 2 or 25, wherein the polypeptide has the amino acid 235H.
28. The polypeptide according to embodiment 2 or 25, wherein the polypeptide has the amino acid 235K.
- 20 29. The polypeptide according to any one of the embodiments 1 and 2-28, wherein the polypeptide comprises an amino acid substitution in position 240.
30. The polypeptide according to embodiment 29, wherein the polypeptide has the amino acid 240E/ H/ M/ D/ S.
31. The polypeptide according to embodiment 29, wherein the polypeptide has the amino acid 240E/ H/ M/ D.
- 25 32. The polypeptide according to embodiment 2 or 29, wherein the polypeptide has the amino acid 240E.

33. The polypeptide according to any one of the embodiments 1-32, wherein the polypeptide has the amino acid 272D.
34. The polypeptide according to any one of the embodiments 1-33, wherein the polypeptide comprises an amino acid substitution in position 311.
- 5 35. The polypeptide according to embodiment 2 or 34, wherein the polypeptide has the amino acid 311P.
36. The polypeptide according to any one of the embodiments 1 and 2-35, wherein the polypeptide comprises an amino acid substitution in position 409.
- 10 37. The polypeptide according to embodiment 36, wherein the polypeptide has the amino acid 409H/ Q/ T/ E.
38. The polypeptide according to embodiment 2 or 37, wherein the polypeptide has the amino acid 409E.
39. The polypeptide according to any one of the embodiments 1-38, wherein the polypeptide has the amino acid 100Q/ K/ N/ R.
- 15 40. The polypeptide according to embodiment 39, wherein the polypeptide has the amino acid 100Q.
41. The polypeptide according to any one of the embodiments 1 and 2-39, wherein the polypeptide has the amino acid 392D/ E/ K/ Y/ N/ Q/ R/ S/ T/ G.
- 20 42. The polypeptide according to embodiment 41, wherein the polypeptide has the amino acid 392D/ E/ K/ Y/ N/ Q/ R/ S/ T.
43. The polypeptide according to embodiment 2 or 41, wherein the polypeptide has the amino acid 392D/ E/ K/ Y.
44. The polypeptide according to embodiment 43, wherein the polypeptide has the amino acid 392D.
- 25 45. The polypeptide according to embodiment 43, wherein the polypeptide has the amino acid 392E.

46. The polypeptide according to embodiment 43, wherein the polypeptide has the amino acid 392K.
47. The polypeptide according to embodiment 43, wherein the polypeptide has the amino acid 392Y.
- 5 48. The polypeptide according to any one of the embodiments 1-47, wherein the polypeptide has both of the following amino acids 235R and 311P.
49. The polypeptide according to any one of the embodiments 1-48, wherein the polypeptide comprises an amino acid substitution in position 16.
- 10 50. The polypeptide according to to embodiment 49, wherein the polypeptide has the amino acid 16/A/E/K.
51. The polypeptide according to any one of the embodiments 1-50, wherein the polypeptide comprises an amino acid substitution in position 48.
52. The polypeptide according to embodiment 51, wherein the polypeptide has the amino acid 48/C/L.
- 15 53. The polypeptide according to any one of the embodiments 1-52, wherein the polypeptide comprises an amino acid substitution in position 97.
54. The polypeptide according to any one of the embodiments 1-53, wherein the polypeptide comprises an amino acid substitution in position 105.
- 20 55. The polypeptide according to embodiment 54, wherein the polypeptide has the amino acid 105/N/R
56. The polypeptide according to any one of the embodiments 1-55, wherein the polypeptide comprises an amino acid substitution in position 248.
57. The polypeptide according to any one of the embodiments 1-56, wherein the polypeptide comprises an amino acid substitution in position 266.
- 25 58. The polypeptide according to any one of the embodiments 1-57, wherein the polypeptide comprises an amino acid substitution in position 347.

59. The polypeptide according to embodiment 58, wherein the polypeptide has the amino acid 347/C/D/H/K.
60. The polypeptide according to any one of the embodiments 1-59, wherein the polypeptide comprises an amino acid substitution in position 350.
- 5 61. The polypeptide according to embodiment 60, wherein the polypeptide has the amino acid 350E/H/N.
62. The polypeptide according to any one of the embodiments 1-61, wherein the polypeptide comprises an amino acid substitution in position 354.
- 10 63. The polypeptide according to embodiment 62, wherein the polypeptide has the amino acid 354D/E.
64. The polypeptide according to any one of the embodiments 1-63, wherein the polypeptide comprises an amino acid substitution in position 362.
65. The polypeptide according to embodiment 64, wherein the polypeptide has the amino acid 362E/H/P.
- 15 66. The polypeptide according to any one of the embodiments 1-65, wherein the polypeptide comprises an amino acid substitution in position 364.
67. The polypeptide according to embodiment 66, wherein the polypeptide has the amino acid 364E/K/NQ.
- 20 68. The polypeptide according to any one of the embodiments 1-67, wherein the polypeptide comprises an amino acid substitution in position 369.
69. The polypeptide according to embodiment 68, wherein the polypeptide has the amino acid 369I/N.
70. The polypeptide according to any one of the embodiments 1-69, wherein the polypeptide comprises an amino acid substitution in position 377.
- 25 71. The polypeptide according to any one of the embodiments 1-70, wherein the polypeptide comprises an amino acid substitution in position 393.

72. The polypeptide according to embodiment 71, wherein the polypeptide has the amino acid 393D/E/K.
73. The polypeptide according to any one of the embodiments 1-72, wherein the polypeptide comprises an amino acid substitution in position 395.
- 5 74. The polypeptide according to embodiment 73, wherein the polypeptide has the amino acid 395C/E/K.
75. The polypeptide according to any one of the embodiments 1-74, wherein the polypeptide comprises an amino acid substitution in position 396.
- 10 76. The polypeptide according to embodiment 75, wherein the polypeptide has the amino acid 396D/E.
77. The polypeptide according to any one of the embodiments 1-76, wherein the polypeptide comprises an amino acid substitution in position 399.
78. The polypeptide according to embodiment 77, wherein the polypeptide has the amino acid 399C/H.
- 15 79. The polypeptide according to any one of the embodiments 1-78, wherein the polypeptide comprises an amino acid substitution in position 400.
80. The polypeptide according to embodiment 79, wherein the polypeptide has the amino acid 400S/W.
- 20 81. The polypeptide according to any one of the embodiments 1-80, wherein the polypeptide comprises an amino acid substitution in position 401.
82. The polypeptide according to embodiment 81, wherein the polypeptide has the amino acid 401D/K.
83. The polypeptide according to any one of the embodiments 1-82, wherein the polypeptide comprises an amino acid substitution in position 403.
- 25 84. The polypeptide according to embodiment 83, wherein the polypeptide has the amino acid 403E/T/V.

85. The polypeptide according to any one of the embodiments 1-84, wherein the polypeptide comprises an amino acid substitution in position 412.
86. The polypeptide according to embodiment 85, wherein the polypeptide has the amino acid 412D/N.
- 5 87. The polypeptide according to any one of the embodiments 1-86, wherein the polypeptide further comprises one or more of the following amino acids 121F, 134R, 141P, 229P, or 307K.
88. The polypeptide according to any one of embodiments 1-87, wherein the polypeptide has one or more of the following amino acids 42K, 88L, 205L, 235R, 240E, 272D,
10 311P, 392D, and 409E.
89. The polypeptide according to any one of embodiments 1-88, wherein the polypeptide comprises the following amino acids 42K, 88L, 235R, 311P, 392D and either 223S, 223K or 223A.
90. The polypeptide according to embodiment 89 further comprising one or more
15 selected from the group consisting of 272D, 409E, 205L or 240E.
91. The polypeptide according to any one of embodiments 1-90, wherein the polypeptide has at least four, five, six, seven or eight of the following amino acids 42K, 88L, 205L, 235R, 240E, 272D, 311P, 392D, or 409E.
92. The polypeptide according to any one of embodiments 1-91, wherein the polypeptide
20 has the amino acid 223A/K/S.
93. The polypeptide according to any one of embodiments 1-92, wherein the polypeptide has the following amino acids 42K, 88L, 223S, 235R, 311P and 392D.
94. The polypeptide according to any one of embodiments 1-93, wherein the polypeptide has the following amino acids 42K, 88L, 223K, 235R, 272D, 311P and 392D.
- 25 95. The polypeptide according to any one of embodiments 1-94, wherein the polypeptide has the following amino acids 42K, 88L, 223S, 235R, 311P, 392D and 409E.

96. The polypeptide according to any one of embodiments 1-95, wherein the polypeptide has the following amino acids 42K, 88L, 205L, 223S, 235R, 311P, 392D and 409E.

97. The polypeptide according to any one of embodiments 1-96, wherein the polypeptide has the following amino acids 42K, 88L, 205L, 223K, 240E, 235R, 311P, 392D and 409E.

98. The polypeptide according to any one of embodiments 1-97, wherein the polypeptide has the following amino acids 42K, 88L, 205L, 223A, T235R, 311P, 392D and 409E.

99. The polypeptide according to any one of embodiments 1-98 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33 and SEQ ID NO: 34.

100. The polypeptide according to any one of embodiments 1-99 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17,

101. The polypeptide according to any one of embodiments 1-100 having an amino acid sequence selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33 and SEQ ID NO: 34, and optionally one or more additional amino acids at the N-terminus.

102. The polypeptide according to embodiment 101 having an amino acid sequence selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17, and optionally one or more additional amino acids at the N-terminus.

103. The polypeptide according to any one of the embodiments 101-102 wherein said one or more additional amino acids at the N-terminus is one M..

104. The polypeptide according to any one of embodiments 1-103, wherein the polypeptide comprises one or more amino acid substitutions at the following

positions: 88, 205, 235 or 409 and/or one or more of the following amino acid substitutions: 42K, 34Q, 100Q, 223A/S, 240E, 311P, 392D, or 409E with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

5 105. The polypeptide according to any one of embodiments 1-104, wherein the polypeptide comprises one or more of the following amino acid substitutions: 42K, 34Q, 88K/L/Y, 100Q, 205L, 223A/S/K, 235K/H/Q/R, 240E/D, 248H, 266T, 311P, 377D/E/P, 392K/D/Y or 409E.

10 106. The polypeptide according to any one of embodiments 1-105, wherein the polypeptide comprises one or more of the following amino acid substitutions: 34Q, 42K, 88L, 100Q, 205L, 223A/S/K, 235K/R, 240E, 311P, 392D or 409E.

107. The polypeptide according to embodiment 106, wherein the polypeptide comprises the following amino acid substitutions: 235R and 311P.

108. The polypeptide according to any one of the embodiments 1-107, wherein the polypeptide comprises the amino acid substitution 42K.

15 109. The polypeptide according to any one of the embodiments 1-108, wherein the polypeptide comprises the following amino acid substitutions: 42K and 223S.

110. The polypeptide according to any one of the embodiments 1-109, wherein the polypeptide comprises the following amino acid substitutions: 42K, 223S and 392D.

20 111. The polypeptide according to any one of the embodiments 1-110, wherein the polypeptide comprises the following amino acid substitutions: 42K, 88L, 223A, 235R, 311P and 392D.

112. The polypeptide according to any one of the embodiments 1-111, wherein the polypeptide comprises the following amino acid substitutions: 42K, 88L, 205L, 223S, 235R, 240E, 311P, 392D and 409E.

25 113. The polypeptide according to any one of the embodiments 1-112, wherein the polypeptide comprises the following amino acid substitutions: 42K, 88L, 205L, 223K, 235R, 240E, 311P, 392D and 409E.

114. The polypeptide according to any one of the embodiments 1-113, wherein the polypeptide comprises the following amino acid substitutions: 42K, 88L, 205L, 223S, 235R, 311P and 392D.
- 5 115. The polypeptide according to any one of the embodiments 1-114, wherein the polypeptide comprises the following amino acid substitutions: 34Q, 42K, 88L, 205L, 223K, 235R, 240E, 311P, 392D and 409E.
116. The polypeptide according to any one of the embodiments 1-115, wherein the polypeptide comprises the following amino acid substitutions: 42K, 88L, 100Q, 205L, 223K, 235R, 240E, 311P, 392D and 409E.
- 10 117. The polypeptide according to any one of embodiments 1-116 further comprising a starch binding domain at the C-terminus.
118. The polypeptide according to embodiment 117 wherein the starch binding domain is the residues of SEQ ID NO: 36.
- 15 119. The polypeptide according to any one of embodiments 1-118 having a linker fused at the C-terminus.
120. The polypeptide according to embodiment 119, wherein the linker is the residues of SEQ ID NO: 35.
121. The polypeptide according to any one of embodiments 1-120 having exoamylase activity.
- 20 122. The polypeptide according to any one of embodiments 1-121 having non-maltogenic exoamylase activity.
123. The polypeptide according to any one of embodiments 1-122, wherein the polypeptide exhibits improved thermo-stability when compared to the SEQ ID NO: 1 or SEQ ID NO: 10.
- 25 124. The polypeptide according to any one of embodiments 1-123, in which the half life ($t_{1/2}$), preferably at 60 degrees C, is increased by 15% or more, preferably 50% or more, most preferably 100% or more, relative to SEQ ID NO: 1 or SEQ ID NO: 10.

125. The polypeptide according to any one of embodiments 1-124, in which a food product treated with the polypeptide has any one or more, preferably all of the following properties: (a) lower firmness; (b) higher resilience; (c) higher cohesiveness; (d) lower crumbliness; and (e) higher foldability compared to a food product which has been treated with SEQ ID NO: 1 or SEQ ID NO: 10.

126. The polypeptide according to any one of embodiments 1-125, in which the resilience, cohesiveness or foldability of the food product treated with the polypeptide is independently increased by 15% or more, preferably 50% or more, most preferably 100% or more, relative to a food product which has been treated with SEQ ID NO: 1 or SEQ ID NO: 10.

127. The polypeptide according to any one of embodiments 1-126, in which each of resilience cohesiveness and foldability of a food product treated with the polypeptide is increased compared to a food product which has been treated with SEQ ID NO: 1 or SEQ ID NO: 10.

128. The polypeptide according to any one of embodiments 1-127, in which the firmness or the crumbliness of the food product treated with the polypeptide is independently decreased by 15% or more, preferably 50% or more, most preferably 100% or more, relative to a food product which has been treated with SEQ ID NO: 1 or SEQ ID NO: 10.

129. The polypeptide according to any one of embodiments 1-128, in which each of the firmness and crumbliness of a food product treated with the polypeptide is decreased compared to a food product which has been treated with SEQ ID NO: 1 or SEQ ID NO: 10.

130. Use of a polypeptide as described in any of embodiments 1-129 as a food or feed additive.

131. A process for treating a starch comprising contacting the starch with a polypeptide as described in any of embodiments 1-129 and allowing the polypeptide to generate from the starch one or more linear products.

132. Use of a polypeptide as described in any of embodiments 1-129 in preparing a food or feed product.

133. A process of preparing a food or feed product comprising admixing a polypeptide as described in any of embodiments 1-129 with a food or feed ingredient.

134. Use according to embodiment 130, or a process according to embodiment 133, in which the food product comprises a dough or a dough product, preferably a processed dough product.

135. A use or process according to any of embodiments 130 to 133, in which the food product is a bakery product.

136. A process for making a bakery product comprising: (a) providing a starch medium; (b) adding to the starch medium a polypeptide as described in any of embodiments 1-129; and (c) applying heat to the starch medium during or after step (b) to produce a bakery product.

137. A food product, feed product, dough product or a bakery product obtained by a process according to any of embodiments 130 to 136.

138. An improver composition for a dough, in which the improver composition comprises a polypeptide as described in any of embodiments 1-129, and at least one further dough ingredient or dough additive.

139. A composition comprising a flour and a polypeptide as described in any of embodiments 1-129.

140. Use of a polypeptide as described in any of embodiments 1-129, in a dough product to retard or reduce staling, preferably detrimental retrogradation, of the dough product.

141. Use of a polypeptide as described in any of embodiments 1-129, in a dough product to improve any one or more of firmness, resilience, cohesiveness, crumbliness or foldability of the dough product.

142. A combination of a polypeptide as described in any of embodiments 1-129, together with any one or more of the following:

- a. maltogenic alpha-amylase also called glucan 1,4- α -maltohydrolase (EC 3.2.1.133) from *Bacillus stearothermophilus*, or a variant, homologue, or mutants thereof which have maltogenic alpha-amylase activity;
- b. a bakery xylanase (EC 3.2.1.8) from e.g. *Bacillus* sp., *Aspergillus* sp.,
5 *Thermomyces* sp. or *Trichoderma* sp.;
- c. α -amylase (EC 3.2.1.1) from *Bacillus amyloliquefaciens* or a variant, homologue, or mutants thereof which have alpha-amylase activity; and
- d. a lipase such as glycolipase from *Fusarium heterosporum*.

- 10 143. Use of a combination according to embodiment 142 for an application according to any preceding embodiment.
144. A food or feed product produced by treatment with a combination according to embodiment 142.
145. A nucleic acid capable of encoding a polypeptide according to any of embodiments 1 to 129.
- 15 146. A nucleic acid according to embodiment 145 having a nucleic acid sequence which at least 78% identical to SEQ ID NO: 52.
147. A nucleic acid comprising a fragment of at least 60 residues of a nucleic acid according to embodiment 145 or 146 which is capable of encoding a polypeptide having non-maltogenic exoamylase activity.
- 20 148. A plasmid comprising a nucleic acid according to any of embodiments 145 to 147.
149. An expression vector comprising a nucleic acid according to any of embodiments 145 to 147, or capable of expressing a polypeptide according to any of embodiments 1 to 129.
- 25 150. A host cell comprising, preferably transformed with, a plasmid according to embodiment 148 or an expression vector according to embodiment 149.

151. A cell capable of expressing a polypeptide according to any of embodiments 1 to 129.

152. A host cell according to embodiment 150, or a cell according to embodiment 151, which is a bacterial, fungal or yeast cell.

5 153. A method of expressing a polypeptide, the method comprising obtaining a host cell or a cell according to embodiment 150, 151 or 152 and expressing the polypeptide from the cell or host cell, and optionally purifying the polypeptide.

10 154. A method of producing a polypeptide according to any of embodiment 1 to 129, the method comprising introducing an amino acid substitution into SEQ ID NO: 1 having non-maltogenic exoamylase activity, the amino acid substitution being

a. in one or more positions selected from the group consisting of: 16, 48, 88, 97, 105, 205, 235, 240, 248, 266, 311, 347, 350, 362, 364, 369, 393, 395, 396, 400, 401, 403, 412 and 409 and/or

15 b. one or more of the following amino acid substitutions: 42K/A/V/N/I/H/F, 34Q, 100Q/K/N/R, 272D, 392 K/D/E/Y/N/Q/R/T/G or 399C/H and/or

c. in one or more positions selected from the group consisting of: 44, 96, 204, 354 and 377 and/or

d. the following amino acid substitution: 392S.

20 155. The polypeptide according to any one of embodiments 1-129 comprising the sequence DGF-X1-AIW-X2-P-X3-PWRD-X4-SSW (SEQ ID NO: 38), wherein X1 is S or T, X2 is M or L, X3 is V or P and X4 is any natural occurring amino acid residue, preferably an L-amino acid, at the positions corresponding to positions 49-66 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

25 156. The polypeptide according to any one of embodiments 1-129 and 155 comprising the sequence GGEGYFW (SEQ ID NO: 39) at the positions corresponding to positions 79-85 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

157. The polypeptide according to any one of embodiments 1-129 and 155-156 comprising the sequence VPNH (SEQ ID NO: 40) at the positions corresponding to positions 114-117 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.
- 5 158. The polypeptide according to any one of embodiments 1-129 and 155-157 comprising the sequence CDDGD (SEQ ID NO: 41) at the positions corresponding to positions 150-154 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.
- 10 159. The polypeptide according to any one of embodiments 1-129 and 155-158 comprising the sequence AGGFRFDFVRG (SEQ ID NO: 42) at the positions corresponding to positions 187-197 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.
- 15 160. The polypeptide according to any one of embodiments 1-129 and 155-159 comprising the sequence FALK (SEQ ID NO: 43) at the positions corresponding to positions 256-259 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.
- 20 161. The polypeptide according to any one of embodiments 1-129 and 155-160 comprising the sequence WREVAVTFVDNHD (SEQ ID NO: 44) at the positions corresponding to positions 282-294 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.
- 25 162. The polypeptide according to any one of embodiments 1-129 and 155-161 comprising the sequence GYSPG (SEQ ID NO: 45) at the positions corresponding to positions 296-300 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.
- 30 163. The polypeptide according to any one of embodiments 1-129 and 155-162 comprising the sequence GQH (SEQ ID NO: 46) at the positions corresponding to positions 304-306 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.
164. The polypeptide according to any one of embodiments 1-129 and 155-163 comprising the sequence AYAYI (SEQ ID NO: 47) at the positions corresponding to positions 318-322 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

165. The polypeptide according to any one of embodiments 1-129 and 155-164 comprising the sequence SPGTP (SEQ ID NO: 48) at the positions corresponding to positions 325-329 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.
- 5 166. The polypeptide according to any one of embodiments 1-129 and 155-165 comprising the sequence VYW (SEQ ID NO: 49) at the positions corresponding to positions 331-333 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.
- 10 167. The polypeptide according to any one of embodiments 1-129 and 155-166 comprising the sequence HMYDWG (SEQ ID NO: 50) at the positions corresponding to positions 335-340 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.
- 15 168. The polypeptide according to any one of embodiments 1-129 and 155-167 comprising the sequence HGGDEIILQFHWN (SEQ ID NO: 37) at the positions corresponding to positions 13-26 with reference to the position numbering of the sequence shown as SEQ ID NO: 1.
- 20 169. A method of producing a polypeptide according to any of embodiments 1 to 129 and 155-167, the method comprising introducing an amino acid substitution into SEQ ID NO: 1 having non-maltogenic exoamylase activity, the amino acid substitution being in one or more of the following positions: 88, or 205 and/or being one or more of the following amino acid substitutions: 42K, 235H/K/R, 240E, 392 K/D/E/Y or 409E.
- 25 170. A method of making a saccharide syrup, comprising adding a polypeptide according to any one of embodiments 1 to 129 and 155-167 to a granular starch liquefact to form the saccharide syrup.
171. The method of embodiment 170, wherein the granular starch liquefact is produced by an alpha-amylase.
172. The method of embodiment 170, wherein the granular starch liquefact is an acid produced liquefact.

173. The method of embodiment 170, wherein the granular starch liquefact is obtained from starch from corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana, or potatoes.
- 5 174. The method of embodiment 170, wherein the granular starch liquefact is saccharified at 55°C to 65°C.
175. The method of embodiment 170, wherein the granular starch liquefact is saccharified at 60°C to 65°C.
176. The method of embodiment 170, wherein the granular starch liquefact is saccharified at pH 5.0 to pH 7.0.
- 10 177. The method of embodiment 170, further comprising a step of adding an enzyme having debranching activity to the granular starch liquefact.
178. The method of embodiment 177, wherein the enzyme having debranching activity is, an isoamylase, a pullulanase, an isopullulanase, a neopullulanase or any combination thereof.
- 15 179. The method of embodiment 177, optionally comprising a further step of adding a protease, a cellulase, a hemicellulase, a lipase, a cutinase, a pectate liase or any combination thereof to the granular starch.
180. The method of embodiment 170, wherein the saccharide syrup comprises at least 35% by weight maltotetraose based on total saccharide content.
- 20 181. The method of embodiment 180, wherein the saccharide syrup comprises at least 45% by weight maltotetraose based on total saccharide content.
182. The method of embodiment 181, wherein the saccharide syrup comprises at least 50% by weight maltotetraose based on total saccharide content.
- 25 183. The method of embodiment 182, wherein the saccharide syrup comprises from 40% by weight to 60% by weight maltotetraose based on total saccharide content.

Examples

Example 1. Cloning of PS4 and G4-amylases

Pseudomonas sacharophila is grown overnight on LB media and chromosomal DNA is isolated by standard methods (Sambrook J, 1989). A 2190 bp fragment containing the PS4 open reading frame (Zhou et al., 1989) is amplified from P. sacharophila chromosomal DNA by PCR using the primers P1 and P2 (see Table 3). The resulting fragment is used as a template in a nested PCR with primers P3 and P4, amplifying the openreading frame of PS4 without its signal sequence and introducing a NcoI site at the 5' end of the gene and a BamHI site at the 3'end. Together with the NcoI site a codon for a N-terminal Methionine is introduced, allowing for intracellular expression of PS4. The 1605 bp fragment is cloned into pCRBLUNT TOPO (Invitrogen) and the integrity of the construct analysed by sequencing. The E.coli Bacillus shuttle vector pDP66K (Penninga et al., 1996) is modified to allow for expression of the PS4 under control of the P32 promoter and the ctgase signal sequence. The resulting plasmid, pCSmta is transformed into B. subtilis.

A second expression construct is made in which the starch binding domain of PS4 is removed. In a PCR with primers P3 and P6 (Table 3) on pCSmta, a truncated version of the mta gene is generated. The full length mta gene in pCSmta is exchanged with the truncated version which resulted in the plasmid pCSmta-SBD.

G4 amylases of Pseudomonas sp. AM1 (2006), Pseudomonas sp. 7193, Pseudomonas mendocina (strain ymp) and Hahella chejuensis (strain KCTC 2396) were cloned into a Bacillus expression vector with signal sequence by gene synthesis (GeneScript; NJ, USA) of the sequences encoding the mature proteins with an M added at the N-terminus as shown in SEQ ID NO 3, 5, 7 and 9.

Example 2. Site-directed mutagenesis

Site-directed mutagenesis was used to produce variant polypeptide as disclosed herein. Mutations were introduced into a nucleic acid encoding pMS 382 having SEQ ID: 1, using the Quick Change™ method (Stratagene, California), according to instructions supplied with the kit with some modifications. Briefly, a single colony was picked and inoculated in 3 ml LB (22 g/l Lennox L Broth Base, Sigma) supplemented with 50 •g/ml kanamycin (Sigma) in a 10 ml Falcon tube. After overnight incubation at 37°C at 200 rpm, the culture was spun down at 5000 rpm for 5 min. The medium was removed and the double-stranded DNA template was prepared using QIAGEN columns (QIAGEN). Primers were designed according to the

manufacturers' protocol. For example, TABLE 1 lists primers that were used to generate new variants based on the nucleotide sequence of pMS382 as shown in SEQ ID NO: 52.

Next, PCR was performed to synthesize the mutant strand. The PCR reaction mix contained the following:

- 5 2.5 •l 10 X QuickChange Multi reaction buffer
- 0.75 •l QuickSolution
- X •l primers (10 pmol for primers of 28-35 nt; 7 pmol for primers of 24-27 nt; or 5 pmol for primers of 20-23 nt)
- 1 •l dNTP mix
- 10 X •l ds-DNA template (200 ng)
- 1 •l QuickChange Multi enzyme blend (2.5 U/•l) (PfuTurbo DNA polymerase)
- X •l dH₂O (to a final volume of 25 •l)

15 The PCR reaction was performed in an Eppendorf thermal cycler for 35 cycles of denaturation (96°C for 1 min), primer annealing (62.8°C for 1 min), and elongation (65°C for 15 min), and then hold at 4°C. For each amplification reaction, 2 •l of DpnI restriction enzyme (10 U/•l) was added, and the mixture was incubated at 37°C for ~3 hr.

20 The DpnI-treated DNA was then used to transform XL10-Gold® Ultracompetent cells (Stratagene). XL10-Gold® cells were thawed on ice. For each mutagenesis reaction, 45 •l cells were added to a pre-chilled Falcon tube. Subsequently, 2 •l of beta-mercaptoethanol mix was added to each tube. The mixture was incubated on ice for 10 min with swirling every 2 min. Then, 1.5 •l DpnI-treated DNA was added to each aliquot of cells, and the mixture was incubated on ice for 30 min. The sample was subject to a heat-pulse of 30 sec at 42°C, and was placed on ice for another 2 min. 0.5 ml of preheated NZY⁺ broth was

25 added to each sample, and incubation was carried at 37°C for 1 hr with shaking at 225-250 rpm. 200 •l of each transformation reaction were plated on LB plates (33.6 g/l Lennox L Agar, Sigma) supplemented with 1% starch and 50 •g/ml kanamycin. The plates were incubated overnight at 37°C. Individual colonies harboring the desired mutations were identified by DNA sequencing and subjected to plasmid preps to harvest plasmids with the

30 desired mutations.

Example 3. Transformation into *Bacillus subtilis*.

Bacillus subtilis (strain DB104A; Smith et al., Gene 70, 351-361 (1988)) is transformed with the mutated plasmid DNA according to the following protocol.

A. Media for protoplasting and transformation

2 x SMM per litre: 342 g sucrose (1 M); 4.72 g sodium maleate (0.04 M); 8.12 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.04 M); pH 6.5 with concentrated NaOH. Distribute in 50-ml portions and autoclave for 10 min.

5 4 x YT (1/2 NaCl) 2 g Yeast extract + 3.2 g Tryptone + 0.5 g NaCl per 100 ml.

SMMP mix equal volumes of 2 x SMM and 4 x YT.

PEG 10 g polyethyleneglycol 6000 (BDH) or 8000 (Sigma) in 25 ml
1 x SMM (autoclave for 10 min.).

B. Media for plating/regeneration

10 agar 4% Difco minimal agar. Autoclave for 15 min.

sodium succinate 270 g/l (1 M), pH 7.3 with HCl. Autoclave for 15 min.

phosphate buffer 3.5 g K_2HPO_4 + 1.5 g KH_2PO_4 per 100ml. Autoclave for 15 min.

MgCl_2 20.3 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ per 100 ml (1 M).

casamino acids 5% (w/v) solution. Autoclave for 15 min.

15 yeast extract 10 g per 100 ml, autoclave for 15 min.

glucose 20% (w/v) solution. Autoclave for 10 min.

DM3 regeneration medium: mix at 60°C (water bath; 500-ml bottle):

250 ml sodium succinate

50 ml casamino acids

20 25 ml yeast extract

50 ml phosphate buffer

15 ml glucose

10 ml MgCl_2

100 ml molten agar

5 Add appropriate antibiotics: chloramphenicol and tetracycline, 5 • g/ml; erythromycin, 1 • g/ml. Selection on kanamycin is problematic in DM3 medium: concentrations of 250 • g/ml may be required.

C. Preparation of protoplasts

Use detergent-free plastic or glassware throughout.

10 Inoculate 10 ml of 2 x YT medium in a 100-ml flask from a single colony. Grow an overnight culture at 25-30°C in a shaker (200 rev/min).

Dilute the overnight culture 20 fold into 100 ml of fresh 2 x YT medium (250-ml flask) and grow until $\text{OD}_{600} = 0.4-0.5$ (approx. 2 h) at 37°C in a shaker (200-250 rev/min).

Harvest the cells by centrifugation (9000 g, 20 min, 4°C).

15 Remove the supernatant with pipette and resuspend the cells in 5 ml of SMMP + 5 mg lysozyme, sterile filtered.

Incubate at 37°C in a waterbath shaker (100 rpm).

20 After 30 min and thereafter at 15 min intervals, examine 25 • l samples by microscopy. Continue incubation until 99% of the cells are protoplasted (globular appearance). Harvest the protoplasts by centrifugation (4000 g, 20 min, RT) and pipet off the supernatant. Resuspend the pellet gently in 1-2 ml of SMMP.

The protoplasts are now ready for use. Portions (e.g. 0.15 ml) can be frozen at -80°C for future use (glycerol addition is not required). Although this may result in some reduction of transformability, 106 transformants per ug of DNA can be obtained with frozen protoplasts).

D. Transformation

Transfer 450 μ l of PEG to a microtube.

Mix 1-10 μ l of DNA (0.2 μ g) with 150 μ l of protoplasts and add the mixture to the microtube with PEG. Mix immediately, but gently.

Leave for 2 min at room temperature, and then add 1.5 ml of SMMP and mix.

- 5 Harvest protoplasts by microfuging (10 min, 13,000 rpm (10,000-12,000 g)) and pour off the supernatant. Remove the remaining droplets with a tissue.

Add 300 μ l of SMMP (do not vortex) and incubate for 60-90 min at 37°C in a waterbath shaker (100 rpm) to allow for expression of antibiotic resistance markers. (The protoplasts become sufficiently resuspended through the shaking action of the waterbath.) Make
10 appropriate dilutions in 1 x SSM and plate 0.1 ml on DM3 plates.

Example 4. Fermentation of Variants in Shake Flasks.

The shake flask substrate is prepared by dissolving the following in water:

	Yeast extract	2% (w/v)
	Soy Flour	2% (w/v)
15	NaCl	0.5% (w/v)
	Dipotassium phosphate	0.5% (w/v)
	Antifoam agent	0.05% (w/v).

The substrate is adjusted to pH 6.8 with 4 N sulfuric acid or sodium hydroxide before autoclaving. 100 ml of substrate is placed in a 500 ml flask with one baffle and autoclaved
20 for 30 minutes. Subsequently, 6 ml of sterile dextrose syrup is added. The dextrose syrup is prepared by mixing one volume of 50% w/v dextrose with one volume of water followed by autoclaving for 20 minutes.

The shake flasks are inoculated with the variants and incubated for 24 hours at 35°C and 180 rpm in an incubator. After incubation cells are separated from broth by centrifugation

(10.000 g in 10 minutes) and finally, the supernatant is made cell free by microfiltration at 0.2 μm . The cell free supernatant is used for assays and application tests.

Table 1. Primers used to generate variants based on the nucleotide sequence of pMS382 as shown in SEQ ID NO: 52.

Mutation	5' Oligo Sequence 3'	modification	Strand	Purpose
Q42K	GTATAACATCCTGAGACAAaaaGCGAGCACAATTGC CG	5' phosphate	+	MSDM
Q42N	GTATAACATCCTGAGACAAaacGCGAGCACAATTGC C	5' phosphate	+	MSDM
R88L	GCATGACTTTAACAAAAACGGCctgTATGGAAGCGA TGCTC	5' phosphate	+	MSDM
R88Y	GGCATGACTTTAACAAAAACGGCtatTATGGAAGCG ATGCTCAAC	5' phosphate	+	MSDM
L88K	GGCATGACTTTAACAAAAACGGCaaaTATGGAAGC GATGCTCAAC	5' phosphate	+	MSDM
S205L	GCCCCGAAAGAGTTGATctgTGGATGAGCGATTCA GCG	5' phosphate	+	MSDM
T235R	GTGGGATTGGAGAAATagaGCGAGCTGGCAGC	5' phosphate	+	MSDM
T235K	GTGGGATTGGAGAAATaaaGCGAGCTGGCAGC	5' phosphate	+	MSDM
T235H	GCCGTGGGATTGGAGAAATcatGCGAGCTGGCAGC AG	5' phosphate	+	MSDM
T235Q	CCGTGGGATTGGAGAAATcagGCGAGCTGGCAGCA G	5' phosphate	+	MSDM
Q240E	CGCTCCAATCTTTGATGATTTCTGCCAGCTCGCTG	5' phosphate	-	MSDM
Q240D	CAGCGAGCTGGCAGgatATCATCAAAGATTGGAGC G	5' phosphate	+	MSDM
Q311P	CAACATAAATGGCCGCTTccgGATGGCCTTATCAGA CAG	5' phosphate	+	MSDM
A392D	GCCCTGAATAGCGATCTGgatAATCCGGGACAAGTT GC	5' phosphate	+	MSDM
A392Y	CGCCCTGAATAGCGATCTGtatAATCCGGGACAAGT TGCTAG	5' phosphate	+	MSDM
S409E	GCGAAGCAGTCAATGCCgaaAATGGCCAAGTCAGA GTCTG	5' phosphate	+	MSDM

Mutation	5' Oligo Sequence 3'	modification	Strand	Purpose
R248H	CATCAAAGATTGGAGCGATcatGCAAAATGCCCCG GTCTTTGAC	5' phosphate	+	MSDM
S266T	CGCATGCAAAATGGAacgGTCGCCGATTGGAAAC ATG	5' phosphate	+	MSDM
S377D	CTACAGTTgatGGCAGCCAACAAAC	5' phosphate	+	MSDM

Example 5. Amylase Assays

5 Betamyl assay

One Betamyl unit is defined as activity degrading 0,0351 mmole per 1 min. of PNP-coupled maltopentaose so that 0,0351 mmole PNP per 1 min. can be released by excess alpha-glucosidase in the assay mix. The assay mix contains 50 ul 50 mM Na-citrate, 5 mM CaCl₂, pH 6,5 with 25 ul enzyme sample and 25 ul Betamyl substrate (Glc5-PNP and alpha-

glucosidase) from Megazyme, Ireland (1 vial dissolved in 10 ml water). The assay mix is incubated for 30 min. at 40°C and then stopped by adding 150 µl 4% Tris. Absorbance at 420 nm is measured using an ELISA-reader and the Betamyl activity is calculated based on Activity = $A_{420} \times d$ in Betamyl units/ml of enzyme sample assayed. For dosing in baking trials 1 BMK = 1000 Betamyl units are used.

Endo-amylase assay

The endo-amylase assay is identical to the Phadebas assay run according to manufacturer (Pharmacia & Upjohn Diagnostics AB).

Exo-specificity

- 10 The ratio of exo-amylase activity to Phadebas activity was used to evaluate exo-specificity.

Example 6. Procedure heat stability determinations based on residual activity

- The enzyme samples in appropriate dilutions are incubated in 50 mM Na-acetate buffer, pH 5.0 with 0.5 M NaCl at room temperature (control) or for 4 minutes at 80°C (heated sample), and immediately after that the control and heated sample are analysed using the Betamyl assay. The residual activity is calculated as the Betamyl activity of the heated sample divided by the Betamyl activity of the control sample.

Example 7. Stabilising effects of mutations

- Using the procedure described in Example 6 the stabilising effects of the mutations Q42K, R88L, S205L, E223S, Q311P, S409E and T235K are shown in Table 2 as an increase in residual activity after heating. Likewise the stabilising effect of A392D is shown in Table 3, of S205L, S223A and S205L combined with S409 in Table 4, of N34Q and G100Q in Table 5 and of Q240E in Table 6. In tables 2-6, the variants have the same sequence as the one mentioned in the first line except for the further mutation(s) made as described in the second column.

- 25 **Table 2.** Stabilising effect of Q42K, R88L, S205L, E223S, Q311P, S409E and T235K combined with Q311P

Variant	Further mutation in pMS382	Residual activity (%)
pMS382 (SEQ ID NO: 1)		8,4
pMS465 (SEQ ID NO: 18)	E223S	9,6

pMS1042 (SEQ ID NO: 19)	Q311P	13,1
pMS1104 (SEQ ID NO: 20)	Q42K	11,2
pMS1153 (SEQ ID NO: 21)	R88L	19,5
pMS1286 (SEQ ID NO: 22)	T235R	12,3
pMS1284 (SEQ ID NO: 23)	T235R, Q311P	17,2
pMS1290 (SEQ ID NO: 24)	T235K	14,1
pMS1484 (SEQ ID NO: 25)	S205L	8,8
pMS1579 (SEQ ID NO: 26)	S409E	16,4

Table 3. Stabilising effect of A392D

Variant	Further mutation in pMS1105	Residual activity (%)
pMS1105 (SEQ ID NO: 27)		11,0
pMS1723 (SEQ ID NO: 28)	A392D	12,3

5 **Table 4.** Stabilising effect of S205L, S223A and S205L combined with S409E

Variant	Further mutation in pMS1776	Residual activity (%)
pMS1776 (SEQ ID NO: 12)		18,9
pMS2104 (SEQ ID NO: 29)	S223A	22,1
pMS2138 (SEQ ID NO: 30)	S205L	22,4
pMS2022 (SEQ ID NO: 15)	S205L, S409E	23,3

Table 5. Stabilising effect of N34Q and G100Q

Variant	Further mutation in pMS2124	Residual activity (%)
pMS2124 (SEQ ID NO: 31)		24,7
pMS2177 (SEQ ID NO: 32)	N34Q	28,8
pMS2178 (SEQ ID NO: 33)	G100Q	26,4

Table 6. Stabilising effect of Q240E

Variant	Further mutation in pMS2022	Residual activity (%)
pMS2022 (SEQ ID NO: 15)		23,3
pMS2118 (SEQ ID NO: 34)	Q240E	26,8

10

Example 10. Recipe for Baking Trials

Baking trials were carried out with a standard white bread sponge and dough recipe for US toast. The sponge dough is prepared from 1500 g of flour "Gold Medal" from General Mills, USA, 890 g of water, 40 g of soy bean oil, 7.5 g GRINDSTED™ SSL P55 Veg, 10 g emulsifier DIMODAN™ PH300, 26 g dry yeast and ascorbic acid (20ppm final concentration). The
5 sponge is mixed for 1 min. at low speed and subsequently 3 min. at speed 2 on a Hobart spiral mixer. The sponge is subsequently fermented for 3 hours at 25 °C, 85% RH.

Thereafter, 500 g of "Gold Medal" flour, 14 g dry yeast, 5 g of calcium propionate, 240 g High Fructose Corn Syrup (42%), 5 g of calcium propionate, 250 g of water and ascorbic acid (30 ppm final concentration) and 40 g of salt are added to the sponge. The resulting dough is
10 mixed for 0.5 min. at low speed and then 10.5 min. on high speed on a Hobart spiral mixer.

The dough is rested for 5 min. at ambient temperature, and then 794 g dough pieces are scaled, moulded on a cross grain moulder and transferred to pans. After 60 min. proofing at 43 °C at 80% RH the doughs are baked for 21 min. at 200 °C in a Reed Rack Oven.

Example 11. Protocol for Evaluation of Firmness, Resilience and Cohesiveness

15 Texture Profile Analysis of Bread

Firmness, resilience and cohesiveness are determined by analysing bread slices by Texture Profile Analysis using a Texture Analyser from Stable Micro Systems, UK. Calculation of firmness, resilience, and cohesiveness is done according to preset standard supplied by Stable Micro System, UK. The probe used is aluminium 50 mm round.

20 Firmness is determined at 40% compression during the first compression. The figure is the force needed to compress the slice to 40% of the total thickness. The lower the value, the softer the bread. The firmness is expressed for example in grams.

Example 12. Evaluation of antistaling effects of G4 amylase variant polypeptides

Bread was baked as described in Example 10 with pMS1776, pMS1934, pMS2020, pMS2022
25 and pMS2062 in dosages varying from 7.5 to 20 BMK/kg corresponding to 7,500 to 20,000 Betamyl units/kg.

The firmness of the bread is tested according to the protocol set out in Example 11 at various times after baking. As controls, bread baked with standard dosages of 600 ppm Novamyl 1500 and/or 416.66 ppm of a composition comprising SEQ ID NO: 1 was also baked.

Fig. 1 and 2 show the firmness results. Relative to the controls with standard dosages 600 ppm Novamyl 1500 and/or 416.66 ppm of a composition comprising SEQ ID NO: 1 a much stronger reduction in the firming rate from day 3 to day 10 after baking is obtained with pMS1776, pMS1934, pMS2020, pMS2022 and pMS2062. Also these breads were strongly improved in resilience and cohesiveness relative to the controls.

This indicates that the variant polypeptide(s) as described herein have highly improved antistaling effects compared to Novamyl 1500 and a composition comprising SEQ ID NO: 1.

Example 13. Protocol for Evaluation of Crumbliness (Resistance to Crumbling)

Two slices of bread are placed on a piece of paper. Each slice is divided into 4 squares by vertical and subsequent horizontal tears of the slice.

Tearing is done by pulling the crumb apart by the fingers. First the slice is torn from the middle of the top bread surface to the middle of the bottom bread surface. Thereafter, each half of the original slice is torn from the crust side to the inside of the slice. The small crumb pieces, which are separated from the 4 squares, are removed by shaking each piece after a tear at least 3 times by moving the hand up and down.

The weight of the separated small crumb pieces is determined as a measure of crumbliness. This assay may be referred to as the "Crumbliness Evaluation Protocol".

Example 14. Protocol for Evaluation of Foldability

The toast bread is sliced using an automatic bread slicer with set slice thickness of 15 mm. The slice is folded by hand from the top of the slice towards the bottom, so that the direction of the crease is from side to side.

The foldability is visually assessed using the following scoring system:

Score	Feature
1	Unfoldable, slice breaks upon folding
2	Foldable, whole slice breaks within 5 seconds after folding

3	Foldable, part of the slice breaks within 5 seconds after folding. Other parts break later.
4	Foldable, part of the slice breaks later than 5 seconds after folding. Other parts do not break.
5	Foldable, no part of the slice break after folding

This assay may be referred to as the "Foldability Evaluation Protocol".

Example 15. Preparation of DP4 syrup

Materials:

SPEZYME FRED® bacterial alpha amylase (Danisco US Inc., Genencor Division) treated
5 granular corn starch liquefact (34%ds, 9.1DE).

Pullulanase (OPTIMAX L-1000, Danisco US Inc., Genencor Division)

Experimental:

Starch liquefact was pH adjusted to 5.2 with NaOH and then each of 100 g liquefact was incubated at 60 C for DP4 saccharification by dosing enzymes at 0.03 BMK/gds.

10 Pullulanase when used was dosed at 0.5 ASPU/gds. Reaction was carried out up to 40 hours, stopped for periodical sampling to check peak DP4. When peak DP4 was observed, the reaction was stopped by heating in boiling water.

Results:

Table 1.

15

Sugar production (%)

Enzymes	Hr	% DP1	% DP2	% DP3	% DP4	% DP5	% DP6	% DP7	% DP8	% DP9	% DP10	% DP11+
pMS2062	16	2.31	5.50	8.67	38.00	0.81	0.17	0.67	0.99	3.23	0.39	39.30
pMS2062+PU		2.42	5.70	8.99	38.12	1.06	0.19	0.85	1.29	4.10	1.06	36.20
pMS2062	19.5	2.49	5.73	9.03	39.86	0.63	0.00	0.51	0.83	2.62	0.38	37.90
pMS2062+PU		2.60	5.89	9.45	40.37	0.88	0.00	0.70	1.18	3.65	1.19	34.10
pMS2062	22	2.59	5.69	9.20	41.16	0.56	0.00	0.45	0.75	2.27	0.37	37.00
pMS2062+PU		2.81	5.95	9.91	41.96	0.78	0.00	0.58	1.02	3.30	1.26	32.40
pMS2062	40	3.41	6.32	10.47	43.76	0.24	0.12	0.16	0.87	0.30	0.20	34.10
pMS2062+PU		3.67	7.01	11.42	45.80	0.43	0.26	0.42	2.22	1.87	0.17	26.70

As shown in Table 1, the results indicated that PMS2062 produced DP4 as a major sugar product. As also indicated, pullulanase (PU) addition (PMS2062 + PU) resulted in faster reduction in higher sugars (see e.g., DP11+), while DP4 level was minimally affected.

SEQUENCES

SEQ ID NO: 1. Mature protein sequence of pMS382

DQAGKSPAGVRYHGGDEIILQGFHWNVREAPYNWYNILRQQASTIAADGFSAIWMPVPWRDFSSWTDGDKSGGGEGYFWHDF
 5 NKNRGYSDAQLRQAAGALGGAGVKVLYDVVPNHMNRFPDKEINLPAGQRFWRNDPDPGNPNDCDDGDRFLGGDEADLNTG
 HPQIYGMFRDEFITNLRSGYGAGGFRDFVRGYAPERVDSWMSDSADSSFCVGEWKEPSEYPPWDWRNTASWQQI IKDWSRA
 KCPVDFDFALKERMQNGSVADWKHGLNGNPDPRWREVAVTFVDNHDITGYSFGQNGGQHKWPLQDGLIRQAYAYILTSPGTPVVY
 WPHMYDWGYGDFIRQLIQVRRTAGVRADSAISFHSYGSLVATVSGSQQTLVVALNSDLANPGQVASGSFSEAVNASNGQVRV
 10 WRS GSGDGGGNDGG

SEQ ID NO: 2. Mature protein of G4 amylase of *Pseudomonas* sp. AM1 (2006) with M added at the N-terminus (Q1EJP2 protein):

1 MDLAGKSPGG VRYHGGDEII LQGFHWNVIR ESPNNWYNIL RDMAPTIAAD
 51 GFSAIWMPVP WRDFSSWSWG ANSGGEGGYF WHDFNKNRGY GSDTQLKQAA
 15 101 GALNNAQVKV LYDVVPNHMNR RGYDPKQINL PAGQGFWRND CADPGNYPND
 151 CDDGDRFMGG DADLNTANPQ VYGMFRDEFA NLRSNYGAGG FRFDFVRGYA
 201 GERVD SWMGA AHDNAFCVGE LWKAPAEYPS WDWNTASWQ QVIKDWSDRA
 251 KCPVDFDFALK ERMQNGSIAD WKNGLNGNPD PRWREVAVTF VDNHDAGYSP
 301 GQNGGQHHWA LQDGLIRQAY AYILTSPGTP VVYWSHMYDW GYGDFIRQLI
 20 351 QVRRTAGVRA DSAISFHSYG SGLVATVSGS QQTLVVALNS DLANPGQVAS
 401 GSFSEAVNAS NGQVRVWRSR SGGGGNDGG EGGLEVNVRN CDNGVTQMGD
 451 SVYAVGNVSQ LGNWSPASAV RLTDITSSYPT WKGSIALPDG QNVEWKCLIR
 501 NEADATLVRRQ WQSGGNNQVQ AAAGASTSGS F*

SEQ ID NO: 3. DNA sequence of nucleotide encoding G4 amylase of *Pseudomonas* sp. AM1 (2006) (Q1EJP2) with M added at the N-terminus (Q1EJP2 DNA):

atggatctggcaggcaaatcaccggggcggttagatatcatggcgcgcatgaaattattctgcaaggtttcattggaatgt
 tattagagaatcaccgaataattgggtataatacactgagagatatggcaccgacaattgcagcagatggcttttcagcaattt
 5 ggatgccggttccgtggagagatttttcatcatggtcagatggcgcaaatcaggcgggcggaaggctatttttggcatgat
 30 tttataaaaaatggcagatatggctcagatacacaactgaaacaagcagcagcgcgactgaataatgcacaagttaaagtctc
 gtatgatgttgttccgaatcatatgaatagaggctatccggataaacaataatctgccggcaggccaaggcttttggagaa
 atgattgcgagatccgggcaattatccgaatgattgcatgatggcgatagatttatggcgcgcatgctgatctgaataca
 gcaaatccgcaagtttatggcatgttagagatgaatttgcaaatctgagatcaaatatggcgcgaggcggtttagatttga
 35 ttttggttagaggctatgcaggcgaaagagttgattcatggatggcgcgagcacatgataatgcattttgcgttggcgaactgt
 ggaaagcaccggcagaatatccgtcatgggattggagaaatcacagcatcatggcaacaagttattaaagattggtcagataga
 gcaaatgcccggtttttttagattttgactgaaagaaagaatgcaaatggctcaattgcagattggaaaaatggcctgaatgg
 caatccggatccgagatggagagaagttgcagttacatttgttgataatcatgatgcaggctattcacggggccaaaatggcg
 40 gccaacatcattgggcactgcaagatggcctgattagacaagcatatgcatatattctgacatcaccgggcacaccggttgtt
 tatttggtcacatatgtatgattggggctatggcgattttattagacaactgattcaagttagaagaacagcaggcggttagagc
 agattcagcaatttcatttcattcaggctattcaggcctggttgcaacagtttcaggctcacaacaacactggttgttgacac
 tgaatagcagatctggcaaatccggggcaagttgcatcaggctcattttcagaagcagttaatgcatcaaatggccaagttaga
 45 gtttggagatcaggctcaggcgatggcgggcgcaatgatggcgcggaaggcggtggttaattgttaatttttagatgcgataa
 tggcggttacacaaatggcgattcagtttatgcagttggcaatgtttcacaactgggcaattggtcaccggcatcagcagttta
 gactgacagatacatcatcatatccgacatgaaaggctcaattgcactgccggatggccaaaatgttgatggaaatgcctg
 attagaaatgaagcagatgcaacactggttagacaatggcaatcaggcgggcaataatcaagttcaagcagcagcaggcgcatc
 aacatcaggctcatttttaa

SEQ ID NO: 4. Mature protein of G4 amylase of *Pseudomonas* sp. 7193 with M added at the N-terminus (A5CVD5 protein):

50 1 MDLAGKSPGG VRYHGGDEII LQGFHWNVIR ESPNNWYNIL RDMAPTIAAD
 51 GFSAIWMPVP WRDFSSWSWG ANSGGEGGYF WHDFNKNRGY GSDTQLKQAA
 101 GALNNAQVKV LYDAVPNHMNR RGYDPKQINL PAGQGFWRND CADPGNYPND
 151 CDDGDRFMGG DADLNTANPQ VYGMFRDEFA NLRSNYGAGG FRFDFVRGYA
 201 GERVD SWMGD GACQRLLRGR ALEGTGRIP E LGLAQYQQLA AVIKDWSDRA
 55 251 KVPGC SNFAL KGAAERLPS PTGRTASTAT PMPRWREVAV TFVDNHDITGY
 301 SPGQNGGQHH WALRDDLVRQ AYAYILASPG TPVVYWSHMY DWGHGFLIRQ
 351 LIQIRRAAGV RADSAIEFHS GYSGLVATVR GTAQTLVMAL GSNLSSPAEV
 401 SSGSFSQALN QDSGQLRIWT TGSTGGDEGD GGGDGTMSV NFRCDNGITQ
 451 PGDSVYAVGS LAQLGSWSPA NAVRLTDVSN YPTWKGAISL PAGQAVEWKC
 60 501 IVRSEADPTQ VRQWQAGDNN RVTAGAGATT IGR L*

SEQ ID NO: 5. DNA sequence of nucleotide encoding [G4 amylase of *Pseudomonas* sp. 7193 (A5CVD5) with M added at the N-terminus (A5CVD5 DNA):

5 atggatctggcaggcgaatcaccggggcggttagatatcatggcgcgcatgaaattattctgcaaggctttcattggaatgt
tattagagaatcaccgaataattggtataatacactgagagatatggcaccgacaattgcagcagatggcttttcagcaattt
ggatgccggttcctggagagatttttcatcatggtcagatggcgcaaatcaggcgggcggaaggctatttttggcatgat
10 tttataaaaaatggcagatatggctcagatacacaactgaaacaagcagcaggcgactgaataatgcacaagttaaagtctt
gtatgatgcagttccgaatcatatgaatagaggctatccggataaacaataatctgccggcaggccaaggcttttgagaa
atgattgcgagatccgggcaattatccgaatgattgcatgtagggcatagatttatggcgcgcatgctgatctgaataca
gcaaatccgcaagtttatggcatgttagagatgaatttgcaaatctgagatcaaatatggcgcgaggcggttttagatttga
15 ttttgtagaggctatgcaggcgaaagagttgattcatggatggcgcatggcgcatgccaagactgctgagaggcagagcac
tggaggcacaggcagaattccggaactgggctggcacaatatggccaactggcagcagttattaaagattggtcagataga
gcaaaagttccgggtgctcaaattttgcactgaaaggcgcatgcagaaagactgccgtcaccgacaggcagaacagcatc
aacagcaacaccgatgccgagatggagagaagttgcagttacatttgttgataatcatgatacaggctattcaccggggccaaa
atggcgggccaacatcattgggcatgagagatgatctggttagacaagcatatgcatatattctggcatcaccggggcacaccg
20 gttgtttatggtcacatatgtatgattggggtcatggaccgctgattagacaactgattcaaatagaagagcagcaggcgt
tagagcagattcagcaattgaatttcattcaggctatccaggcctggttgcaacagtttagaggcacagacaaaactgggtta
tggcactgggctcaaatctgtcatcaccggcgagaagtttcattcaggctcattttcacaagcactgaataagattcaggccaa
ctgagaatttggaacaacaggctcaacaggcgcgcatgaaggcgatggcgggcgcatggcacaatggtttcagttaattttag
atgcgataatggcattacacaaccggggcgattcagtttatgcagttggctcactggcacaactgggtcatggtcaccggcaa
25 atgcagttagactgacagatgtttcaaatatccgacatggaaaggcgcaatttcactgccggcaggccaagcagttgaatgg
aatgcattgttagatcagaagcagatccgacacaagtttagacaatggcaagcaggcgataataatagagttacagcaggcg
aggcgcaacaacaattggcagactgtaa

SEQ ID NO: 6. Mature protein of G4 amylase of *Pseudomonas mendocina* (strain ymp) with M added at the N-terminus (A4XX23 protein):

25 1 MDAPGKTASG VRYHGGDEII LQGFHWNTVR TSSNWIYATLA SMAPTAAADG
51 FSAIWMPVPW RDFSSWSDPG NGTSGGGEY FWHDFNKNR YGSDSLLRQA
101 ASALNAAGVK PIYDVVPNHM NRGYPDKIN LPAGQGLWRH DCNDPGNYAN
151 DDDGDRFMG GDADLNTGHP QNYAMFRDEF ARLRSQYGAG GFRFDFVRGY
30 201 AGERVASWMS DAHDNGFCLG ELWKAPGEYP SWDWRNGASW QQILKDWSR
251 AKCTVDFDAL KERMONGGIA DWRHGLNGNP DARWREAVT FVDNHDITGYS
301 PGPHGGQHHW PLPDARLKQA YAYILSSPGT PVVYWPHMYD WGHGDFIRQL
351 IQIRRAAGVK AASAIQFHTG FSGLVATISG SQQLLLIALD SNLSSPGQVA
401 SGDFTQALNT DNGAIRIWSR GQGGGDGQGN LVSVNFRCDN GVTQWGDVSY
35 451 ALGNVTQLGN WSPAGAVRLT DTSAYPTWKG SIALPAGQV QWKCIVRSES
501 NPTQVKTWQP GGNSVTVAS GASTAGSF*

SEQ ID NO: 7. DNA sequence of nucleotide encoding G4 amylase of *Pseudomonas mendocina* (strain ymp) (A4XX23) with M added at the N-terminus: (A4XX23 DNA):

40 atggatgcaccgggcaaacagcatcaggcggttagatatcatggcgcgcatgaaattattctgcaaggctttcattggaatac
agttagaacatcatcaattggtatgcaacactggcatcaatggcaccgacactggcagcagatggcttttcagcaatttgga
tgccggttcctggagagatttttcatcatggtcagatccgggcaatggcacatcaggcgggcggaaggctattttggcat
gattttaataaaaaatggcagatatggctcagattcactgctgagacaagcagcatcagcactgaatgcagcaggcggttaaacc
gatttatgatgttctccgaatcatatgaatagaggctatccggataaagaaattaatctgccggcaggccaaggcctgtgga
45 gacatgattgcaatgatccgggcaattatgcaaatgattgcatgatggcgatagatttatggcgcgcatgctgatctgaat
acaggccatccgcaaaattatgcaatgttagagatgaatttgcaagactgagatcacaatatggcgaggcggtttagatt
tgattttgttagaggctatgcaggcgaaagagttgcatcatggatgtcagatgcacatgataatggcttttgctggcggaac
tgtggaaagcaccggcgcaat atccgtcatgggattggagaaatggcgcatcatggcaacaatctggaagattggtcagat
agagcaaaatgcacagtttttgattttgcactgaaagaaagaatgcaaatggcgcatatgcagattggagacatggcctgaa
50 tggcaatccggatgcaagatggagagaagttgcagttacatttgttgataatcatgatacaggctattcaccggggcccgcatg
gcggccaacatcattggccgctgccggatgcaagactgaaacaagcatatgcatatattctgtcatcaccggggcacaccggtt
gtttattggcgcatatgtatgattggggtcatggagattttattagacaactgattcaaatagaagagcagcaggcgttaa
agcagcatcagcaattcaatttcatacaggcttttcaggcctggttgcaacaatttcagggtcacaacaacaactgctgattg
cactggattcaaatctgtcatcaccggggcaagttgcatcaggcgattttacacaagcactgaatacagataatggcgcaatt
55 agaatttgagatcaggccaaggcgcgcatggccaaggcaatctggtttcagtttaattttagatgcgataatggcggttac
acaatggggcgattcagtttatgcaactgggcaatgttacacaactgggcaattggtcaccggcaggcgagtttagactgacag
atacatcagcatatccgacatggaaaggctcaattgcaactggcgaggccaacaagttcaatggaaatgcattgttagatca
gaatcaaatccgacacaagttaaaacatggcaaccggggcggaataattcagttacagttgcatcaggcgcatcaacagcagg
60 ctcattttaa

SEQ ID NO: 8. Mature protein of G4 amylase of *Hahella chejuensis* (strain KCTC 2396) with M added at the N-terminus (Q2SEA8 protein):

65 1 MESSGKSGAG VRFHGGDEII LQGFHWNVVR TAERNWYNIL QSKAQQISED
51 GFTAIWMPVP WRDNSSWQAS SDTRFGGEY FWADMDKNSR YGDDGQLKQA
101 ASALKNKGVK VIYDIVPNHH DRGHSNDSL NPSGQGYRS DCSSCDDGDP

5
151 FMDGGSDFST AHPDVYDLFK NELVNLKTNV SAGGFRFDFV RGYAPERISA
201 WMSASLDSGY CVGELWKGPS EYPSWDWRHS ASWQEILKDF TDASDCSVFD
251 FALKERMONG SISDWRYGLN GNPSAQWREV AVTFVDNHD TGYSPGPLGGQ
301 HHWALPDWKR KMAYAYILSS PGTPVYVWPH MYDWGMRDFI RNLIQLRKSA
351 GVKAYSGVQF HDGFSGLVGT TSGSNGKLLF AIDS NFSSPN QVAGGAWNLA
401 VNEDNGRIRI WRQ*

10 SEQ ID NO: 9. DNA sequence of nucleotide encoding of G4 amylase of *Hahella*
chejuensis (strain KCTC 2396) with M added at the N-terminus (Q2SEA8 DNA):
atggaatcatcaggcaaatcaggcgcaggcgtagatttcatggcggcgatgaaattattctgcaaggtttcattggaacgt
tgtagaacagcagaaagaaactggtacaacatcctgcaatcaaaagcacaacaaatttcagaagatggctttacagcaattt
ggatgccggttccgtggagagataattcatcatggcaagcatcatcagatacaagatttggcggcgaaggctatttttgggca
15 gatatggataaaaattcaagatatggcgatgatggccaactgaacaagcagcatcagcactgaaaaataaaggcgtaaagt
tatttatgatattgttccgaatcatcatgatagagccattcaaatgattcactgaatctgccgtcaggccaaggctattata
gatcagattgctcatcatgcgatgatggcgatccggttatggatggcggctcagatttttcaacagcacatccggatgtttac
gatctgtttaaaaacgaactggttaacctgaaaacaaactactcagcaggcggttttagattttgattttgttagaggctatgc
accggaagaattttcagcatggatgtcagcatcactggattcaggctattgcgttggcgaactgtggaaaggcccgtcagaat
20 atccgtcatgggattggagacattcagcatcatggcaagaaattctgaaagattttacagatgcacagattgctcagttttt
gattttgcactgaaagaaagaatgcaaaatggctcaatttcagattggagatatggcctgaatggcaatccgtcagcacaatg
gagagaagttgcagttacatttgttgataatcatgatacaggctattcaccgggcccgtggcgcccaacatcattgggcac
tgccgatttgaaaagaaaaatggcatatgcatatattctgtcatcaccgggcacaccggttggtttattggccgcatatgtat
gattggggcatgagagattttattagaaatctgattcaactgagaaaaatcagcaggcggttaaagcatattcaggcggttcaatt
25 tcatgatggcttttcaggcctggttggcacaacatcaggctcaaatggcaaaactgctgtttgcaattgattcaaatttttcat
caccgaatcaagttgcaggcggcgcatggaatctggcagttaatgaagataatggcagaattagaatttggagacaataa

30 SEQ ID NO: 10. >gi|77787|pir||S05667 glucan 1, 4-alpha-maltotetraohydrolase (EC
3.2.1.60) mature protein without the signal sequence - *Pseudomonas saccharophila*
DQAGKSPAGVRYHGGDEIILQGFHWNVVREAPNDWYNILRQQASTIAADGFSAIWMPVPWRDSSWTDGGKSGGGEGYFWHDF
NKNRGYGSDAQLRQAAGALGGAGVKVLYDVVPNHMNRGYPDKINLPAGQGFWRNDCADPGNYPNDCDDGDRFIGGESDLNTG
HPQIYGMFRDELANLRSGYGAGGFRDFVRGYAPERVD SWMSDSADSSFCVGE LWKGPSEYP SWDWRNTASWQQIIKDWSDRA
KCPVDFDALKERMONGSVADWKHGLNGNPDPRWREVAVTFVDNHD TGYS PGQNGGQHHWALQDGLIRQAYAYILTSPGTPVYV
WSHMYDWGYGDFIRQLIQVRRTAGVRADSAISFHSGYSLVATVSGSQTLVVALNSDLANPGQVASGSFSEAVNASNGQVRV
35 WRSGSGDGGGNDGEGGLVNVNFRCDNGVTQMGSVYAVGNVSQLGNWSPASAVRLTDTSSYPTWKGSIALPDGQNVWEWKCLI
RNEADATLVRQWQSGGNNQVQAAAGASTSGSF

40 SEQ ID NO: 11. Signal peptide of glucan 1, 4-alpha-maltotetraohydrolase (EC
3.2.1.60) - *Pseudomonas saccharophila*
MSHILRAAVLA AVL LFPFALA

SEQ ID NO: 12. Mature protein sequence of pMS1776
1 DQAGKSPAGV RYHGGDEIIL QGFHWNVVRE APYNWYNILR QKASTIAADG
51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HGFNKNGLYG SDAQLRQAAG
45 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNPNDC
151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDVVRGYAP
201 ERVDSWMSDS ADSSFCVGE LWKSPSEYPPW DWRNRASWQQ IIKDWSDRAK
251 CPVDFDALKERMQNGSVADW KHGLNGNPDPRWREVAVTFV DNHD TGYS PG
301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWP MYDWG YGDFIRQLIQ
50 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 13. Mature protein sequence of pMS1934
1 DQAGKSPAGV RYHGGDEIIL QGFHWNVVRE APYNWYNILR QKASTIAADG
55 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HGFNKNGLYG SDAQLRQAAG
101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNPNDC
151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDVVRGYAP
201 ERVDSWMSDS ADSSFCVGE LWKSPSEYPPW DWRNRASWQQ IIKDWSDRAK
251 CPVDFDALKERMQNGSVADW KDGLNGNPDPRWREVAVTFV DNHD TGYS PG
60 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWP MYDWG YGDFIRQLIQ
351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

65 SEQ ID NO: 14 Mature protein sequence of pMS2020
1 DQAGKSPAGV RYHGGDEIIL QGFHWNVVRE APYNWYNILR QKASTIAADG

51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGLYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVIRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKSPSEYPPW DWRNRASWQQ IIKDWSRAK
 5 251 CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 401 SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

10 SEQ ID NO: 15 Mature protein sequence of **pMS2022**

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGLYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVIRGYAP
 15 201 ERVDLWMSDS ADSSFCVGEL WKSPSEYPPW DWRNRASWQQ IIKDWSRAK
 251 CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 401 SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

20 SEQ ID NO: 16 Mature protein sequence of **pMS2062**

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGLYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 25 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVIRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKSPSEYPPW DWRNRASWQE IIKDWSRAK
 251 CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 30 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 17 Mature protein sequence of **pMS2171**

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGLYG SDAQLRQAAG
 35 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVIRGYAP
 201 ERVDLWMSDS ADSSFCVGEL WKAPSEYPPW DWRNRASWQQ IIKDWSRAK
 251 CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ
 40 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 401 SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 18 Mature protein sequence of **pMS465:**

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQASTIAADG
 45 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVIRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKSPSEYPPW DWRNTASWQQ IIKDWSRAK
 251 CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 50 301 QNGGQHKWPL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 19 Mature protein sequence of **pMS1042:**

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQASTIAADG
 55 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVIRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKSPSEYPPW DWRNTASWQQ IIKDWSRAK
 60 251 CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 20 Mature protein sequence of pMS1104:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 5 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGAGF RFDFVRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKEPSEYPPW DWRNTASWQQ IIKDWSDRAK
 251 CPVDFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 10 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 21 Mature protein sequence of pMS1153:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 15 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGLYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGAGF RFDFVRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKEPSEYPPW DWRNTASWQQ IIKDWSDRAK
 251 CPVDFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 20 301 QNGGQHKWPL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 22 Mature protein sequence of pMS1286:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGAGF RFDFVRGYAP
 30 201 ERVDSWMSDS ADSSFCVGEL WKEPSEYPPW DWRNRASWQQ IIKDWSDRAK
 251 CPVDFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 35 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 23 Mature protein sequence of pMS1284:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG
 40 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGAGF RFDFVRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKEPSEYPPW DWRNRASWQQ IIKDWSDRAK
 251 CPVDFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ
 45 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 24 Mature protein sequence of pMS1290:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGAGF RFDFVRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKEPSEYPPW DWRNKASWQQ IIKDWSDRAK
 55 251 CPVDFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL QDGLIRQAYA YILTSPGTPV VYWPAMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 25 Mature protein sequence of pMS1484:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 65 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGAGF RFDFVRGYAP

5 201 ERVDLWMSDS ADSSFCVGEL WKEPSEYPPW DWRNTASWQQ IIKDWSDRAK
 251 CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 26 **Mature protein sequence of pMS1579:**

10 1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QQASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGAGF RFDFVIRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKEPSEYPPW DWRNTASWQQ IIKDWSDRAK
 251 CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 15 301 QNGGQHKWPL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

20 SEQ ID NO: 27 **Mature protein sequence of pMS1105:**

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 251 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGAGF RFDFVIRGYAP
 25 201 ERVDSWMSDS ADSSFCVGEL WKSPSEYPPW DWRNTASWQQ IIKDWSDRAK
 251 CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LANPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 28 **Mature protein sequence of pMS1723:**

35 1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGRYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGAGF RFDFVIRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKSPSEYPPW DWRNTASWQQ IIKDWSDRAK
 251 CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL QDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 40 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 29 **Mature protein sequence of pMS2104:**

45 1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGLYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGAGF RFDFVIRGYAP
 201 ERVDSWMSDS ADSSFCVGEL WKAPSEYPPW DWRNRASWQQ IIKDWSDRAK
 50 251 CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

55 SEQ ID NO: 30 **Mature protein sequence of pMS2138:**

60 1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEGYFW HDFNKNGLYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNGPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSYGAGAGF RFDFVIRGYAP
 201 ERVDLWMSDS ADSSFCVGEL WKSPSEYPPW DWRNRASWQQ IIKDWSDRAK
 251 CPVFDFALKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 65 401 SFSEAVNASN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 31 Mature protein sequence of pMS2124:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 5 51 FSAIWMPVPW RDFSSWTDGD KSGGGEYFW HDFNKNGLYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVRGYAP
 201 ERVDLWMSDS ADSSFCVGEL WKKPSEYPPW DWRNRASWQE IIKDWSDRAK
 251 CPVDFDFAKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 10 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 401 SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 32 Mature protein sequence of pMS2177:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYQWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEYFW HDFNKNGLYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVRGYAP
 20 201 ERVDLWMSDS ADSSFCVGEL WKKPSEYPPW DWRNRASWQE IIKDWSDRAK
 251 CPVDFDFAKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 401 SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 33 Mature protein sequence of pMS2178:

1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEYFW HDFNKNGLYG SDAQLRQAAG
 30 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVRGYAP
 201 ERVDLWMSDS ADSSFCVGEL WKKPSEYPPW DWRNRASWQE IIKDWSDRAK
 251 CPVDFDFAKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 35 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 401 SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

SEQ ID NO: 34 Mature protein sequence of pMS2118:

40 1 DQAGKSPAGV RYHGGDEIIL QGFHWNVRE APYNWYNILR QKASTIAADG
 51 FSAIWMPVPW RDFSSWTDGD KSGGGEYFW HDFNKNGLYG SDAQLRQAAG
 101 ALGGAGVKVL YDVVPNHMNR FYDPKEINLP AGQRFWRNDC PDPGNPNDC
 151 DDGDRFLGGE ADLNTGHPQI YGMFRDEFTN LRSGYGAGGF RFDFVRGYAP
 201 ERVDLWMSDS ADSSFCVGEL WKSPSEYPPW DWRNRASWQE IIKDWSDRAK
 45 251 CPVDFDFAKE RMQNGSVADW KHGLNGNPDP RWREVAVTFV DNHDTGYSPG
 301 QNGGQHKWPL PDGLIRQAYA YILTSPGTPV VYWPHMYDWG YGDFIRQLIQ
 351 VRRTAGVRAD SAISFHSGYS GLVATVSGSQ QTLVVALNSD LDNPGQVASG
 401 SFSEAVNAEN GQVRVWRSGS GDGGGNDGG*

50 SEQ ID NO: 35. Linker Sequence
 GSGDGGGNDGG

SEQ ID NO: 36: Sequence of PS4 SBD:

55 1 EGGLVNVNFR CDNGVTQMGD SVYAVGNVSQ LGNWSPASAV RLTDTSYPT
 51 WKGSIALPDG QNVEWKCLIR NEADATLVRQ WQSGGNNQVQ AAAGASTSGS
 101 F*

60 SEQ ID NO: 37
 HGGDEIILQFHWN

SEQ ID NO: 38
 DGF-X1-AIW-X2-P-X3-PWRD-X4-SSW

SEQ ID NO: 39
GGEGYFW

5 SEQ ID NO: 40
VPNH

SEQ ID NO: 41
CDDGD

10 SEQ ID NO: 42
AGGFRFDFVRG

SEQ ID NO: 43
FALK

15 SEQ ID NO: 44
WREVAVTFVDNHD

20 SEQ ID NO: 45
GYSPG

SEQ ID NO: 46
GQH

25 SEQ ID NO: 47
AYAYI

SEQ ID NO: 48
SPGTP

30 SEQ ID NO: 49
VYW

35 SEQ ID NO: 50
HMYDWG

SEQ ID NO: 51: Novamyl:

SSSASVKGDVIYQIIIDRFYDGDITNNNPAKSYGLYDPTKSKWKMYWGGDLEGVRQKLPYLKQLGVTTIWLSPLVDNLDTLAG
40 TDNTGYHGYWTRDFKQIEEHFGNWTTFDTLVNDAHQNGIKVIVDFVPNHSTPFKANDSTFAEGGALYNNGTYMGNYFDDATKG
YFHHNGDISNWDRIYAQWKNFTDPAGFSLADLSQENGITIAQYLDAAVQLVAHGADGLRIDAVKHFNSGFSKSLADKLYQKK
DIFLVGEWYGDDPGTANHLEKVRYANN SGVNVLD FDLNTVIRNVFGTFTQTM YDLNNMVNQ TGNEYKYKENLITFIDNHMSR
FLSVNSNKANLHQALAFILTSRGTPSIYYGTEQYMAGGNDPYNRGMPAFDTTTTAFKEVSTLAGLRRNNAAIQYGTITQRWI
NNDVYIYERKFFNDVVLVAINRNTQSSYSISGLQTALPNGSYADYLSGLLGNGISVSNNGSVASFTLAPGAVSVWQYSTSASA
45 PQIGSVAPNMGIPGNVVTIDGKGF GTTQGT VTFGGVTATVKS WTSNRIEVYVPNMAAGLTDVKVTAGGVSSNLYSYNLSGTQ
TSVVF TVKSAPPTNLGDKIYLTGNIPELGNWSTDTSGAVNNAQGPLLAPNYPDFYVFSVPAGKTIQFKFFIKRADGTIQWEN
GSNHVATTPTGATGNITVTWQN

SEQ ID NO: 52: Nucleotide sequence of pMS382:

1 gatcaagcag gaaaaagccc ggcaggcgctc agatatcatg gcggcgatga aatcatcctt
50 61 cagggtcttcc attggaacgt cgctcagagaa gcgcggtata actggtataa catectgaga
121 caacaagcga gcacaattgc cgctgatggc ttttcgcga tctggatgcc ggttcctgtg
181 agagatttta gcagctggac ggatggagat aaaagcggag gcggcggaagg atatttttgg
241 catgacttta acaaaaacgg ccgctatgga agcgatgctc aactgagaca agcagcagga
301 gcacttggag gagcaggagt caaagtcctg tacgatgtcg tcccgaacca tatgaaccgc
55 361 ttttatccgg acaaagaaat caatctgccg gcaggccaaa gattttggag aaacgattgc
421 ccggaccggg gaaatggacc gaatgattgc gatgatggcg atagatttct gggcggcgaa
481 gcggatctga atacaggcca tccgcaaatac tatggcatgt ttcgggacga atttacgaat
541 ctgagaagcg gatatggagc gggcggtatt cgctttgatt ttgtcagagg ctatgccccg
601 gaaagagttg atagctggat gagcgattca gcggatagca gcttttgctt cggcgaaactt
661 tggaaagaac cgagcgaata tccgcctgtg gattggagaa atacagcgag ctggcagcag
721 atcatcaaag attggagcga tagagcaaaa tgcccgtctt ttgactttgc cctgaaagaa
781 cgcattgcaaa atggaagcgt cgccgattgg aaacatggcc tgaacggaaa tccggaccgg
841 agatggagag aagtcgccgt cacgtttgtc gataaccatg acacaggata tagcccgagg
901 caaaatggag gacaacataa atggccgctt caagatggcc ttatcagaca ggcgtatgcc
65 961 tatatcctta catcaccggg aacaccggtt gtttattggc cgcataatgta tgattggggc
1021 tatggcgatt tcatccgcca actgatccag gttagaagaa cagcaggagt cagagcggat

1081 agcgccatta gctttcatag cggctatagc ggacttgctg ctacagttag cggcagccaa
1141 caaacactgg tcgtcgccct gaatagcgat ctggcaaata cgggacaagt tgctagcggc
1201 agcttttagcg aagcagtcaa tgccagcaat ggccaagtca gagtctggag aagcggaagc
1261 ggagatggag gaggaaatga cggaggataa

5

Note: The derived N-terminus of this nucleotide sequence is DQA... However, the final construct may be extended at the 5' end with atg to encode M at the N-terminus.

CLAIMS

1. A polypeptide having amylase activity comprising an amino acid sequence having

a. at least 78 % sequence identity to the amino acid sequence of SEQ ID NO: 1,
and wherein the polypeptide comprises one or more amino acid substitutions
at the following positions: 235, 16, 48, 97, 105, 240, 248, 266, 311, 347,
350, 362, 364, 369, 393, 395, 396, 400, 401, 403, 412 or 409 and/or

b. at least 65 % sequence identity to the amino acid sequence of SEQ ID NO: 1,
and wherein the polypeptide comprises one or more amino acid substitutions
at the following positions: 88 or 205, and/or

c. at least 78 % sequence identity to the amino acid sequence of SEQ ID NO: 1,
and wherein the polypeptide comprises one or more of the following amino
acid substitutions: 42K/A/V/N/I/H/F, 34Q, 100Q/K/N/R, 272D, 392
K/D/E/Y/N/Q/R/T/G or 399C/H and/or

d. at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1,
and wherein the polypeptide comprises one or more amino acid substitutions
at the following positions: 44, 96, 204, 354 or 377 and/or

e. at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1,
and wherein the polypeptide comprises the following amino acid substitution:
392S

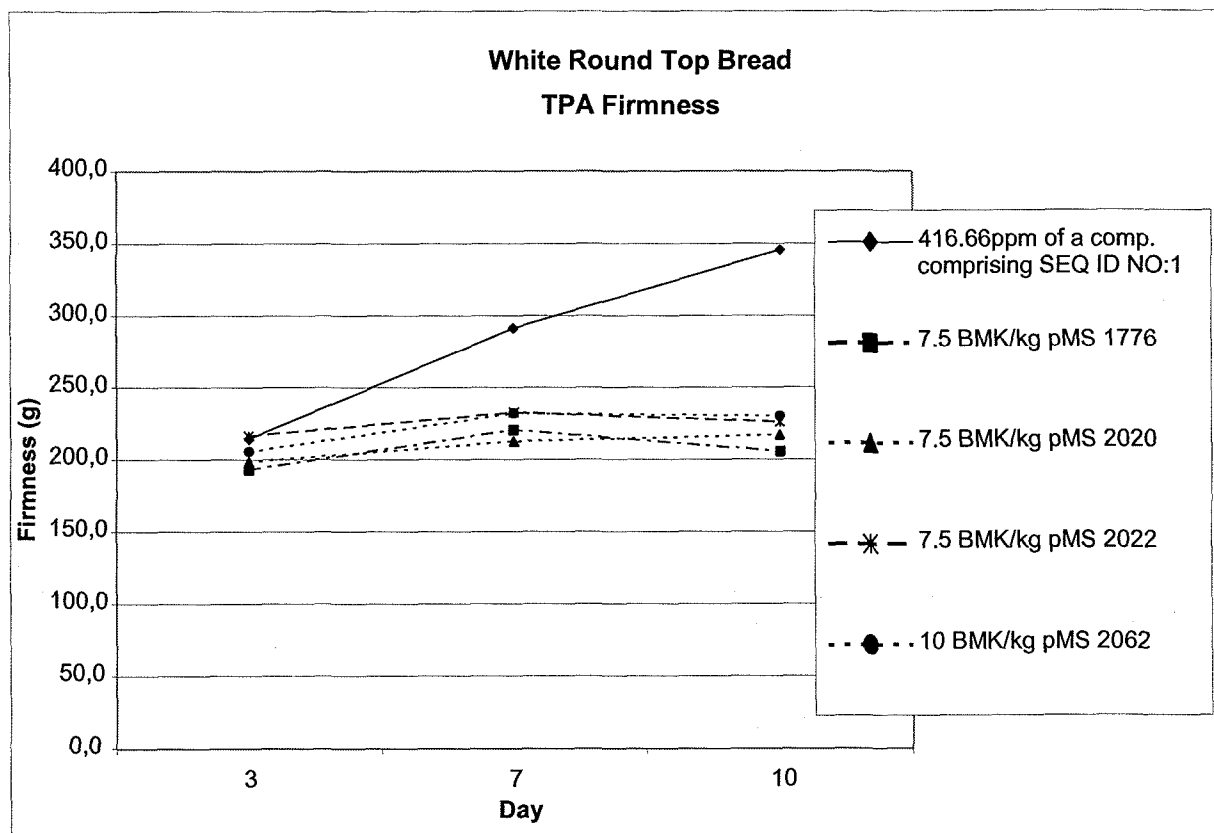
with reference to the position numbering of the sequence shown as SEQ ID NO: 1.

2. The polypeptide according to claim 1, wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 235, 88, 205, 240, 248, 266, 311, 377 or 409 and/or one or more of the following amino acid substitutions: 42K/A/V/N/I/H/F, 34Q, 100Q/K/N/R, 272D or 392K/D/E/Y/N/Q/R/S/T/G.

3. The polypeptide according to any one of claims 1-2, wherein the polypeptide comprises one or more amino acid substitutions at the following positions: 235, 88, 205, 240, 311 or 409 and/or one or more of the following amino acid substitutions: 42K/N/I/H/F, 272D, or 392 K/D/E/Y/N/Q/R/S/T/G.

4. The polypeptide according to any one of claims 1-3, wherein the polypeptide comprises amino acid substitutions at least in four, five or in all of the following positions: 88, 205, 235, 240, 311 or 409 and/or has at least one or two of the following amino acid substitutions: 42K/N/I/H/F, 272D or 392 K/D/E/Y/N/Q/R/S/T/G.
- 5 5. The polypeptide according to any one of claims 1-4, wherein the polypeptide further comprises one or more of the following amino acids 33Y, 34N, 70D, 121F, 134R, 141P, 146G, 157L, 161A, 178F, 179T, 223E/S/K/A, 229P, 307K, 309P and 334P.
6. The polypeptide according to any one of claims 1-5 having at least 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity to the amino acid sequence of SEQ ID NO: 1.
- 10 7. The polypeptide according to any one of the claims 1-6, wherein the polypeptide comprises an amino acid substitution in position 88.
8. The polypeptide according to claim 7, wherein the polypeptide has the amino acid 88L.
9. The polypeptide according to any one of the claims 1-8, wherein the polypeptide comprises an amino acid substitution in position 235.
- 15 10. The polypeptide according to claim 9, wherein the polypeptide has the amino acid 235R.
11. The polypeptide according to any one of the claims 1-10, wherein the polypeptide further comprises one or more of the following amino acids 121F, 134R, 141P, 229P, or 307K.
- 20 12. The polypeptide according to any one of claims 1-11 comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33 and SEQ ID NO: 34.
- 25 13. The polypeptide according to any one of claims 1-12 having a linker fused at the C-terminus.
14. The polypeptide according to any one of claims 1-13 having exoamylase activity.

15. The polypeptide according to any one of claims 1-14 having non-maltogenic
exoamylase activity.
16. Use of a polypeptide according to any of claims 1-15 as a food or feed additive.
17. A method of making a saccharide syrup, comprising adding a polypeptide according to
5 any of claims 1-15 to a granular starch liquefact to form the saccharide syrup.

**Fig. 1**

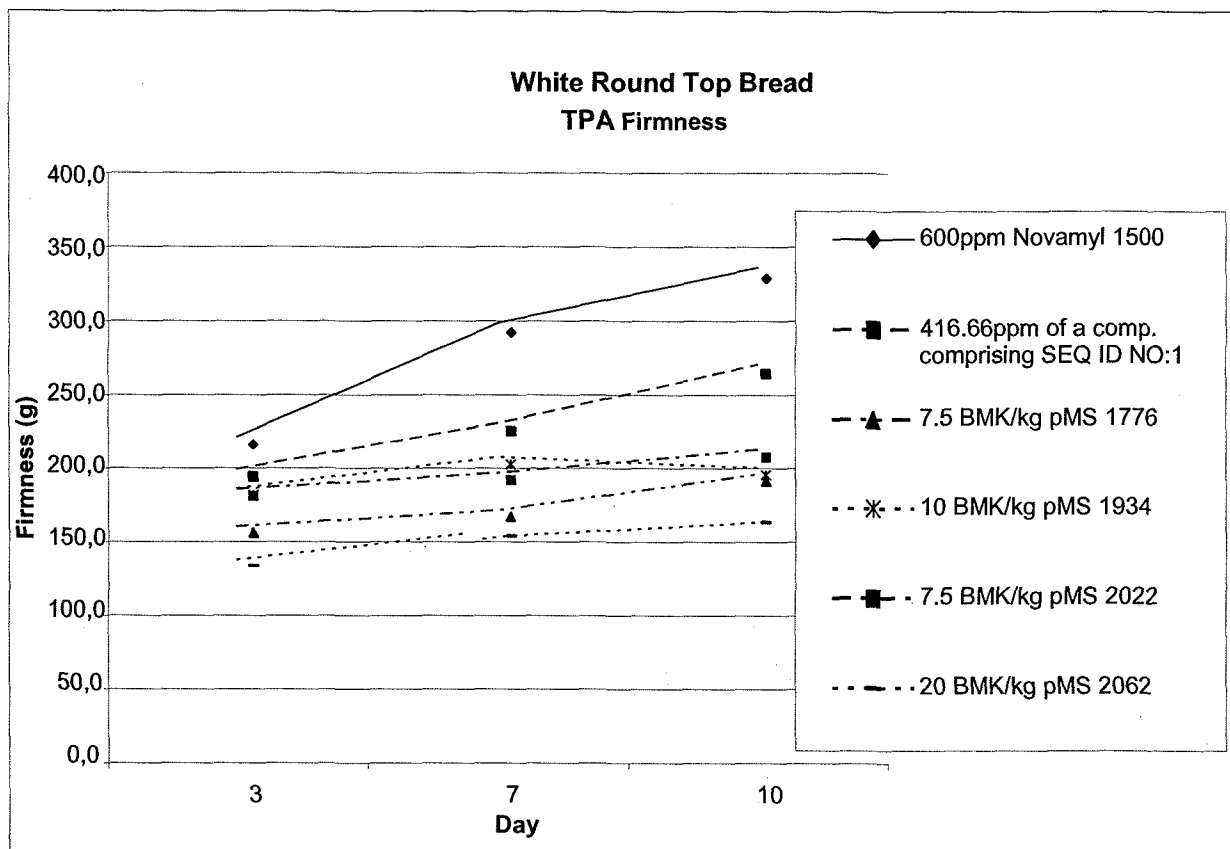


Fig. 2