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(54) **Title:** ALICYCLOBACILLUS POHLIAE ALPHA-AMYLASE VARIANTS

(57) **Abstract:** The present invention describes a variant polypeptide having alpha-amylase activity and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity. Such a variant polypeptide may be used in the food industry such as for example in the preparation of a baked product.



WO 2016/030448 A1

ALICYCLOBACILLUS POHLIAE ALPHA-AMYLASE VARIANTS

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Field of the invention

The invention relates to a variant polypeptide having alpha-amylase activity. The invention also relates to a nucleic acid sequence encoding such a variant, to a recombinant expression vector a said nucleic acid construct and to a recombinant host cell comprising a said expression vector. Further, the invention relates to a method for producing an alpha amylase via use of such a host cell. Also, the invention relates to a method of producing an alpha-amylase polypeptide variant. The invention further relates to a composition comprising an alpha-amylase variant, to use of such an alpha-amylase variant or alpha-amylase variant-containing composition in the preparation of a baked product, to a process for the production of a baked product and to the resulting baked product.

Background of the invention

20 In bread making starch plays a major role in the crumb formation and the rate of crumb staling of the baked bread. In dough starch is present as granules, absorbing only a small amount of water. During baking the starch gelatinization process is taking place. Amylose is leaking out of the granule and forms a continuous gel in the baking dough. Already during baking part of the amylose is re-crystallizing, resulting in stiffening of the gel and setting of the crumb. At the same time water is entering the granule and hydrating the amylopectin resulting in swelling of the granule. During storage of the bread over several days, the amylopectin starts to re-crystallize (also called retrogradation). The staling of bread is believed to be a direct reflection of the retrogradation of amylopectin. The starch and thus the breadcrumb become more rigid.

30 The firmness of bread after a certain storage time is depending on the initial softness, which is the softness after cooling down, and the rate of increase of firmness, the rate of staling.

Studies on bread staling have indicated that the starch fraction in bread recrystallizes during storage, thus causing an increase in crumb firmness, which may be measured as an increase in hardness of bread slices.

The present invention relates to an alpha-amylase. Alpha-amylases have
5 been used in industry for a long time.

Alpha-amylases have traditionally been provided through the inclusion of malted wheat or barley flour and give several advantages to the baker. Alpha-amylase is used to give satisfactory gas production and gas retention during dough leavening and to give satisfactory crust color. This means that if this enzyme is not used in sufficient
10 amount, the volume, texture, and appearance of the loaf are substantially impaired. Alpha-amylase occurs naturally within the wheat crop itself, measured routinely by Hagberg Falling Number (ICC method 107), and steps are taken to minimise such variations by the addition of alpha-amylase at the mill and through the use of specialty ingredients at the bakery as the enzyme is of such critical importance.

15 In more recent times, alpha-amylase from cereal has been largely replaced with enzymes from microbial sources, including fungal and bacterial sources. Through use of biotechnology in strain selection, fermentation and processing, enzymes can be prepared from such microbial sources and this brings advantage over malt flour because the enzyme is of more controlled quality, relatively pure and more cost effective in use.

20 The properties of alpha-amylases, and their technological effects, do however show important differences. Besides giving influence to gas production, gas retention and crust color, alpha-amylase can have bearing on the shelf-life of the baked product.

Starch within the wheat flour contains two principal fractions, amylose and amylopectin, and these are organised in the form of starch granules. A proportion of
25 these granules from hard-milling wheat varieties become "damaged", with granules splitting apart as a consequence of the energy of milling. In the process of baking, the starch granules gelatinise; this process involves a swelling of the granule by the uptake of water and a loss of the crystalline nature of the granule; in particular amylopectins within the native granule are known to exist as crystallites and these molecules
30 dissociate and lose crystallinity during gelatinisation. Once the bread has been baked, amylopectin recrystallises slowly over a numbers of days and it is this recrystallisation, or retrogradation of starch, that is regarded as being the principal cause of bread staling.

These varying forms of the starch and their interaction with alpha-amylase dictate the role the enzyme has with respect to baking technology.

Alpha-amylase from fungal sources, most typically coming from *Aspergillus* species, acts principally on damaged starch during the mixing of dough and throughout fermentation/proof. The low heat stability of the enzyme means that the enzyme is inactivated during baking and, critically before starch gelatinisation has taken place, such that there is little or no breakdown of the starch from the undamaged fraction. As a consequence, fungal amylase is useful in providing sugars for fermentation and color, but has practically no value in extending shelf-life. Bacterial alpha-amylase, most typically from *Bacillus amyloliquifaciens*, on the other hand does bring extended temperature stability and activity during the baking of bread and while starch is undergoing gelatinisation. Bacterial amylase then leads to more extensive modification of the starch and, in turn, the qualities of the baked bread; in particular the crumb of the baked bread can be perceptibly softer throughout shelf-life and can permit the shelf-life to be increased. However, while bacterial alpha-amylase can be useful with regard to shelf-life extension, it is difficult to use practically as small over-doses lead to an unacceptable crumb structure of large and open pores, while the texture can become too soft and "gummy".

There is a need for an alpha-amylase with improved performance in industry, especially in the baking industry.

US 4,598,048 describes the preparation of a maltogenic amylase enzyme. US 4,604,355 describes a maltogenic amylase enzyme, preparation and use thereof. US RE38,507 describes an antistaling process and agent. WO99/43793 discloses amylolytic enzyme variants. WO99/43794 maltogenic alpha-amylase variants. WO2004/081171 discloses an enzyme. WO2006/012899 discloses maltogenic alpha-amylase variants. US8426182 discloses an alpha amylase. WO2008/148845 discloses a method for preparing a dough. WO2006/032281 discloses a method for preparing a dough.

Description of the Figures

Figure 1. Sets out the plasmid map of pDBC1, the plasmid is used to construct the expression vectors for alpha-amylase variants. The *BsmBI* sites are used to exchange the CAP marker for a promoter fragment and gene of interest. The region between the *amyE* flanking regions integrated integrates in the *amyE* locus and integrands are selected on spectinomycin.

Figure 2. Sets out the plasmid map of pDBC-AM1 containing the G01 expression module and the *DSM-AM* gene that is used for the production of a reference alpha-amylase.

Description of the sequence listing

5

SEQ ID NO: 1 sets out the polynucleotide sequence from *Alicyclobacillus pohliae* NCIMB14276 encoding the wild type signal sequence (set out in nucleotides 1 to 99), the wild-type alpha-amylase polypeptide (set out in nucleotides 100 to 2157), and a stop codon at the 3'-terminus (set out in nucleotides 2157 to 2160).

10

SEQ ID NO: 2 sets out the amino acid sequence of the mature *Alicyclobacillus pohliae* NCIMB14276 wild type alpha-amylase polypeptide.

SEQ ID NO: 3 sets out a synthetic DNA fragment containing a *TthIII* site, 340 bp of 5'-amyE, *BsmBI* site, chloramphenicol selection marker, terminator, *BsmBI* site, lox 66 site, spectinomycin selection marker, lox71 site, 120 bp of 3'-amyE and *AsiI* site.

15

SEQ ID NO: 4 sets out a synthetic DNA fragment of the G01 expression module which contains a P15 promoter, a modified RNA leader sequence as described in EP2186880 (nucleotides 31-251 of SEQ ID NO: 70 therein) a *NdeI* site at the ATG start and two *BsmBI* sites at the 5' and 3' ends.

20

SEQ ID NO: 5 sets out the nucleic acid sequence that contains a *BsmBI* restriction, which is placed at the 5' end of the DNA sequences encoding the alpha-amylase variants.

SEQ ID NO 6: sets out the nucleic acid sequence that contains a *BsmBI* restriction, which is placed at the 3' end of the DNA sequences encoding the alpha-amylase variants.

25

SEQ ID NO 7: sets out the polynucleotide sequence of a synthetic DNA construct containing a *BsmBI* site, wild type *DSM-AM* sequence as set out in SEQ ID NO: 1, double stop codon and *BsmBI* restriction site.

Summary of the invention

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The invention relates to variant polypeptides having alpha-amylase activity, i.e. to alpha-amylase variants. An alpha-amylase variant of the invention may have one or more improved properties in comparison with a reference polypeptide, the reference polypeptide typically having alpha-amylase activity. A reference polypeptide may be a

wild-type alpha-amylase, such as wild-type alpha-amylase, for example from *Alicyclobacillus pohliae*, in particular *Alicyclobacillus pohliae* NCIMB14276 strain. The reference polypeptide having alpha-amylase activity may be the polypeptide as set out in SEQ ID NO: 2.

5 The improved property will typically be a property with relevance to the use of the variant alpha-amylase in the preparation of a baked product.

 The improved property may include:
one or more of:

- a) an increased thermostability;
- 10 b) an increased sucrose tolerance;
- c) an increased thermostability in the presence of sucrose
- d) an increased Activity at pH4 : Activity at pH5 ratio;
- e) an increased Activity at pH7 : Activity at pH5 ratio; and
- f) an increased specific activity,
- 15 as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The improved property may include

- g) an increased ratio of the activity at a temperature of 50 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

25 The ratio of the activity at a temperature of 50 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions is herein also referred to as the Activity at 50°C or higher: Activity at 37°C ratio.

30 According to the invention there is thus provided a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15,

124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity.

The invention also provides:

- 5 - a nucleic acid sequence encoding a variant of the invention;
- a nucleic acid construct comprising such a nucleic acid sequence operably linked to one or more control sequences capable of directing the expression of an alpha-amylase in a suitable expression host;
- a recombinant expression vector comprising such a nucleic acid construct; and
- 10 - a recombinant host cell comprising such an expression vector.

The invention also relates to a method for producing an alpha-amylase comprising cultivating the host cell of the invention under conditions conducive to production of the alpha-amylase and recovering the alpha-amylase.

Further the invention relates to:

- 15 - a composition or pre-mix comprising the variant of the invention or obtainable by a method of the invention;
- use of a variant alpha-amylase according to the invention or of a composition of the invention in the preparation of a dough and/or a baked product;
- a dough comprising the variant of the invention or obtainable by a method of
- 20 the invention;
- a process for the production of a baked product, which method comprises comprising adding an effective amount of a variant polypeptide according to the invention of a composition according to the invention to dough and carrying out appropriate further baking manufacturing steps.; and
- 25 - a baked product obtainable by such process or use.

Also, the invention relates to a method of producing an alpha-amylase polypeptide variant, which method comprises:

- a) selecting an alpha-amylase polypeptide;
 - 30 b) substituting at least one amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue corresponding to any of amino acids
- 14, 15, 124, 133, 225, 200, 282, 358,
said positions being defined with reference to SEQ ID NO: 2;

- c) optionally substituting one or more further amino acids as defined in b);
 - d) preparing the variant resulting from steps a)-c);
 - e) determining a property of the variant; and
 - f) selecting a variant having an altered property in comparison to the polypeptide
- 5 of a), thereby to produce an alpha-amylase polypeptide variant.

Detailed description of the invention

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

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to
15 one or at least one) of the grammatical object of the article. By way of example, "an
element" may mean one element or more than one element.

Alpha-amylase activity can suitably be determined using the Ceralpha®
procedure, which is recommended by the American Association of Cereal Chemists
20 (AACC). A variant of the invention will typically retain alpha-amylase activity. That is to
say, a variant of the invention will typically be capable of alpha amylase activity. The
reference polypeptide as set out in SEQ ID NO: 2 showed alpha-amylase activity in the
CERALPHA assay.

A variant of the invention will typically be a starch degrading enzyme.

25 Variant polypeptides of the invention may be referred to as "variant polypeptide
having alpha-amylase activity", "alpha-amylase variant", "improved alpha-amylase", and
the like.

A gene or cDNA coding for an alpha-amylase or pro-alpha-amylase, for example
a variant of the invention, may be cloned and over-expressed in a host organism. Well
30 known host organisms that have been used for alpha amylase over-expression in the
past include *Aspergillus*, *Kluyveromyces*, *Trichoderma*, *Escherichia coli*, *Pichia*,
Saccharomyces, *Yarrowia*, *Neurospora* or *Bacillus*.

The alpha-amylase variant may be manufactured industrially using recombinant
DNA technology, e.g. using filamentous fungi such as *Aspergillus* species, yeast strains,

e.g. of *Kluyveromyces* species, or bacterial species, e.g. *E. coli*, as host organisms. Such recombinant microbial production strains are constructed and continuously improved using DNA technology as well as classical strain improvement measures directed towards optimising the expression and secretion of a heterologous protein.

5 In the invention, an alpha-amylase variant may be provided in the form of prealpha-amylase variant or (mature) alpha-amylase variant. A corresponding nucleic acid sequence may also be provided, i.e. a polynucleotide that encodes a pre-alpha-amylase or a (mature) alpha-amylase may be provided.

10 Herein, positions which may be substituted to achieve a variant of the invention are defined with reference to SEQ ID NO: 2 which is a mature alpha-amylase, i.e. it is a sequence which does not include a presequence.

The invention concerns variant polypeptides having alpha-amylase activity as compared with a reference polypeptide having alpha-amylase activity. The reference polypeptide may typically be a wild-type polypeptide having alpha-amylase activity, such as the alpha-amylase of SEQ ID NO: 2. The reference polypeptide may also be referred to as a parent polypeptide or comparison polypeptide.

15 In an embodiment the variant polypeptide according to the invention is a variant having alpha-amylase activity, wherein the variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 200, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

25 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha amylase activity as set out in SEQ ID NO: 2.

In an aspect the one or more altered properties are selected from an increased thermostability, an increased sucrose tolerance, an increased an increased Activity at pH4 : Activity at pH5 ratio and an increased Activity at pH7 : Activity at pH5 ratio.

30 In an embodiment the variant polypeptide according to the invention is a variant having alpha-amylase activity, wherein the variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 and at least one further substitution

of an amino acid residue corresponding to amino acid 200, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha amylase activity as set out in SEQ ID NO: 2 selected
5 from an increased thermostability, an increased sucrose tolerance, an increased an increased Activity at pH4 : Activity at pH5 ratio and an increased Activity at pH7 : Activity at pH5 ratio.

In an embodiment the variant polypeptide according to the invention is a variant having
10 alpha-amylase activity, wherein the variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue corresponding to amino acid 200, said positions being defined
15 with reference to SEQ ID NO: 2;

and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha amylase activity as set out in SEQ ID NO: 2 selected from an improved crumb softness of the baked product, improved resilience both initial and in particular after storage, reduced hardness after storage and improved anti-staling
20 of the baked product.

In an embodiment the variant polypeptide according to the invention is a variant having alpha-amylase activity, wherein the variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when
25 aligned with the sequence as set out in SEQ ID NO: 2, comprises the amino acid substitutions W70Y and S200N, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha amylase activity as set out in SEQ ID NO: 2 selected
30 from an increased thermostability, an increased sucrose tolerance, an increased an increased Activity at pH4 : Activity at pH5 ratio and an increased Activity at pH7 : Activity at pH5 ratio.

In an embodiment the variant polypeptide according to the invention is a variant having alpha-amylase activity, wherein the variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises the amino acid substitutions W70Y and S200N, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha amylase activity as set out in SEQ ID NO: 2 selected from an improved crumb softness of the baked product, improved resilience both initial and in particular after storage, reduced hardness after storage and improved anti-staling of the baked product.

In an embodiment the variant polypeptide according to the invention is a variant having alpha-amylase activity, wherein the variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue corresponding to amino acid 200, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant demonstrates a reduced loss of resilience over storage of a baked product comprising at least 2 wt% sugar, in an aspect comprising at least 3 wt% sugar, in an aspect comprising at least 5 wt% sugar, compared with a reference polypeptide having alpha amylase activity as set out in SEQ ID NO: 2.

In an embodiment the variant polypeptide according to the invention is a variant having alpha-amylase activity, wherein the variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises the amino acid substitutions W70Y and S200N, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant demonstrates a reduced loss of resilience over storage of a baked product comprising at least 2 wt% sugar, in an aspect comprising at least 3 wt% sugar, in an aspect comprising at least 5 wt% sugar, compared with a reference polypeptide having alpha amylase activity as set out in SEQ ID NO: 2.

In an embodiment the variant polypeptide according to the invention is a variant having alpha-amylase activity, wherein the variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue corresponding to amino acid 200, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant demonstrates a reduced hardness after storage of a baked product comprising at least 2 wt% sugar, in an aspect comprising at least 3 wt% sugar, in an aspect comprising at least 5 wt% sugar, compared with a reference polypeptide having alpha amylase activity as set out in SEQ ID NO: 2.

In an embodiment the variant polypeptide according to the invention is a variant having alpha-amylase activity, wherein the variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises the amino acid substitutions W70Y and S200N, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant demonstrates a reduced hardness after storage of a baked product comprising at least 2 wt% sugar, in an aspect comprising at least 3 wt% sugar, in an aspect comprising at least 5 wt% sugar, compared with a reference polypeptide having alpha amylase activity as set out in SEQ ID NO: 2.

More concretely, the invention relates to a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue corresponding to any of amino acids

14, 15, 124, 133, 225, 200, 282, 358,

said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity.

A wild type reference polypeptide may be obtained from any suitable organisms.

Suitable wild type reference polypeptides may be obtained from *Alicyclobacillus pohliae* NCIMB14276.

Preferably, the reference polypeptide is the alpha amylase set out in SEQ ID NO: 2.

The parent polypeptide having alpha-amylase activity is preferably is the alpha amylase set out in SEQ ID NO: 2.

According to the invention, there is thus provided a variant polypeptide having
5 alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue corresponding to any of amino acids

10 14, 15, 124, 133, 225, 200, 282, 358,

said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

An altered property includes an improved property.

15 A variant polypeptide will typically have an improved property as compared to a reference polypeptide, in particular with respect to a property relevant to the use of the variant polypeptide in baked product making.

Improved productivity may be demonstrated by an alpha-amylase variant that shows improved expression as compared with a parent polypeptide.

20 The improved property will typically be a property with relevance to the use of the variant alpha-amylase in the preparation of a baked product.

An alpha-amylase variant with an improved property relevant for preparation of a baked product may demonstrate reduced hardness after storage of a baked product and/or reduced loss of resilience over storage of a baked product.

25 The improved property may include increased strength of the dough, increased elasticity of the dough, increased stability of the dough, reduced stickiness of the dough, improved extensibility of the dough, improved machineability of the dough, increased volume of the baked product, improved flavour of the baked product, improved crumb structure of the baked product, improved crumb softness of the baked product, reduced
30 blistering of the baked product, improved crispiness, improved resilience both initial and in particular after storage, reduced hardness after storage and/or improved anti-staling of the baked product.

The improved property may include an improved oven spring.

The improved property may include an improved slice-ability.

The improved property may include an improved crumbliness.

The improved property may include an improved slice-stickiness.

The improved property may include faster dough development time of the dough and/or reduced dough stickiness of the dough.

5 The improved property may include improved foldability of the baked product, such as improved foldability of a tortilla, a pancake, a flat bread, a pizza crust, a roti and/or a slice of bread.

 The improved property may include improved flexibility of the baked product including improved flexibility of a tortilla, a pancake, a flat bread, a pizza crust, a roti
10 and/or a slice of bread.

 The improved property may include improved stackability of flat baked products including tortillas, pancakes, flat breads, pizza crusts, roti.

 The improved property may include reduced stickiness of noodles and/or increased flexibility of noodles.

15 The improved property may include reduced clumping of cooked noodles and/or improved flavor of noodles even after a period of storage.

 The improved property may include reduction of formation of hairline cracks in a product in crackers as well as creating a leavening effect and improved flavor development.

20 The improved property may include improved mouth feel and /or improved softness on squeeze,

 The improved property may include reduced damage during transport, including reduced breaking during transport.

 The improved property may include reduced hardness after storage of gluten-free
25 bread.

 The improved property may include improved resilience of gluten-free bread. The improved property may include improved resilience both initial and in particular after storage of gluten-free bread.

 The improved property may include reduced hardness after storage of rye bread.

30 The improved property may include reduced loss of resilience over storage of rye bread.

 The improved property may include reduced loss of resilience over storage of a baked product comprising at least 2 wt% sugar, in an aspect comprising at least 3 wt% sugar, in an aspect comprising at least 5 wt% sugar, in an aspect at least 8 wt% sugar,

in an aspect comprising at least 12 wt% sugar, in an aspect comprising at least 15 wt% sugar based on total recipe weight. In an aspect comprising at least 18 wt% sugar, in an aspect comprising at least 20 wt% sugar, in an aspect comprising at least 25 wt% sugar, in an aspect comprising at least 30 wt% sugar based on total recipe weight. Herein 5 wt%
5 sugar means 50 grams sugar per 1000 grams total recipe weight etc..

The improved property may include reduced hardness after storage of a baked product comprising at least 2 wt% sugar, in an aspect comprising at least 3 wt% sugar, in an aspect comprising at least 5 wt% sugar, in an aspect comprising at least 8 wt% sugar, in an aspect comprising at least 12 wt% sugar, in an aspect comprising at least
10 15 wt% sugar based on total recipe weight. In an aspect comprising at least 18 wt% sugar, in an aspect comprising at least 20 wt% sugar, in an aspect comprising at least 25 wt% sugar, in an aspect comprising at least 30 wt% sugar based on total recipe weight. Herein 5 wt% sugar means 50 grams sugar per 1000 grams of total recipe weight, etc.

15 Each of these improvements may be determined as compared with a reference polypeptide. The improved property may be demonstrated by preparing a baked product comprising the alpha-amylase variant and another comprising a parent polypeptide and comparing the results.

A variant which exhibits a property which is improved in relation to the parent
20 polypeptide having alpha-amylase activity is one which demonstrates a measurable reduction or increase in the relevant property, typically such that the variant is more suited to use as set out below, for example in a method for the production of a foodstuff.

The improved property may be demonstrated in an assay or (bio)chemical analysis.

25 In particular, a variant alpha-amylase of the invention may show improved productivity in comparison with a reference polypeptide. Alternatively, or in addition, a variant alpha-amylase of the invention may show an altered, such as reduced or increased, temperature stability or an altered activity at pH relevant for the baked product making process, such as a lower pH or a higher pH, as compared with a
30 reference polypeptide having alpha-amylase activity.

The improved property may include one or more of:

- an increased thermostability, for example at pH 4, pH 5, pH 6 and/or pH 7;
- an increased specific activity at pH 4, pH 5, pH 6 and/or pH 7;
- an increased sucrose tolerance for example at pH 4, pH 5, pH 6 and/or pH 7;

- an increased stability/activity at different pH range;
- an increased activity on raw starch;
- an altered temperature optimum;
- an altered substrate specificity and
- 5 - an increased productivity in the production of the alpha-amylase variant, as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO :2).

The improved property may include one or more of:

- 10 a) an increased thermostability;
- b) an increased sucrose tolerance;
- c) an increased thermostability in the presence of sucrose
- d) an increased Activity at pH4 : Activity at pH5 ratio;
- e) an increased Activity at pH7 : Activity at pH5 ratio; and
- 15 f) an increased specific activity,
- as compared to a reference polypeptide having alpha-amylase activity as set out in SEQ ID NO: 2.

The improved property may include

- 20 g) an increased ratio of the activity at a temperature of 50 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.
- 25 h) The improved property may include an increased ratio of the activity at a temperature of 60 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.
- 30 i) The improved property may include an increased ratio of the activity at a temperature of 60 degrees Celsius versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.
- j) The improved property may include an increased ratio of the activity at a temperature of 80 degrees Celsius versus the activity at a temperature of 37

degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

- 5 The ratio of the activity at a temperature of 60 degrees Celsius versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions is herein also referred to as the Activity at 60°C: Activity at 37°C ratio.

- 10 The ratio of the activity at a temperature of 80 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions is herein also referred to as the Activity at 80°C: Activity at 37°C ratio.

- 15 Table 1 sets out positions that may influence specific properties of the variant alpha-amylases of the invention.

- 20 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises the amino acid substitution W70Y and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

- 25 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises the amino acid substitution W70Y and comprises a substitution of an amino acid residue corresponding to one or more of

- 30 I14V, I15V, V124I, S133T, S200N, L225F, L282I, L282F and S358A, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises the amino acid substitutions W70Y and S200N, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

- W70Y and S200N;
- W70Y and L282F;
- W70Y and L282I;
- W70Y and S133T;
- W70Y and L225F;
- W70Y and I14V;
- W70Y and I15V;
- W70Y and S358A;
- W70Y and V124I;
- W70Y, S200N and L225F;
- W70Y, L282F and L225F;
- W70Y, L282I and L225F;
- W70Y, S133T and L225F;
- W70Y, S200N and S358A;
- W70Y, L282F and S358A;
- W70Y, L282I and S358A;
- W70Y, S133T and S358A;
- W70Y, S200N and V124I;
- W70Y, L282F and V124I;
- W70Y, L282I and V124I;
- W70Y, S133T and V124I;
- W70Y, S200N and S133T;
- W70Y, L282F and S133T;

W70Y, L282I and S133T;
W70Y, S200N, S133T and L225F;
W70Y, L282F, S133T and L225F;
W70Y, L282I, S133T and L225F;
W70Y, S200N, S133T and S358A;
W70Y, L282F, S133T and S358A;
W70Y, S200N, S133T and V124I;
W70Y, L282F, S133T and V124I;

said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

As is known to the skilled person W70Y and S200N means 2 substitutions: the amino acid at position 70 and the amino acid at position 200 are substituted. The W (Tryptophan) at position 70 is changed into a Y (Tyrosine) and that S (Serine) at position 200 is changed into a N (Asparagine), etc.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 and at least one further substitution of an amino acid residue corresponding to any of amino acids

14, 15, 124, 133, 200, 282, 358,

said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises the amino acid substitution L225F and comprises a substitution of an amino acid residue corresponding to one or more of 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

In an embodiment the variant polypeptide according to the invention is a variant having alpha-amylase activity, wherein the variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha amylase activity as set out in SEQ ID NO: 2.

In an embodiment the variant polypeptide according to the invention is a variant having alpha-amylase activity, wherein the variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha amylase activity as set out in SEQ ID NO: 2 selected from an increased thermostability, an increased sucrose tolerance, an increased an increased Activity at pH4 : Activity at pH5 ratio and an increased Activity at pH7 : Activity at pH5 ratio.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises the amino acid substitution L225F and comprises a substitution of an amino acid residue corresponding to one or more of I14V, I15V, V124I, S133T, S200N, L282I, L282F and S358A,

said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of the amino acid residues corresponding to amino acids 225 and 133 or 200, said positions being defined with reference to SEQ ID NO: 2.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises the amino acid substitutions L225F and S200N, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

- W70Y and L225F;
- W70Y, S200N and L225F;
- W70Y, L282F and L225F;
- W70Y, L282I and L225F;
- W70Y, S133T and L225F;
- W70Y, S200N, S133T and L225F;
- W70Y, L282F, S133T and L225F;
- W70Y, L282I, S133T and L225F;
- L225F and I14V;
- L225F and I15V;
- L225F and S358A;
- L225F and V124I;
- L225F, S358A and I14V;
- L225F, V124I and I15V;
- S200N, L225F and I14V;
- S200N, L225F and I15V;
- S200N, L225F and S358A;
- S200N, L225F and V124I;

S200N, L225F, S358A and I14V;
S200N, L225F, V124I and I15V;
S200N, L282F, S133T and L225F;
S200N, L282I, S133T and L225F;
S200N, L282F, S133T, L225F and S358A;
S200N, L282I, S133T, L225F and S358A;
S200N, L282F, S133T, L225F and V124I; or
S200N, L282I, S133T, L225F and V124I,

said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

- 5 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

W70Y and S200N
W70Y and L282F
W70Y and L282I
W70Y and L225F
W70Y and I14V
W70Y and I15V
W70Y and S358A
W70Y and V124I
W70Y, S200N and L225F
W70Y, L282F and L225F
W70Y, L282I and L225F
W70Y, S133T and L225F
W70Y, S200N and S358A
W70Y, L282F and S358A
W70Y, L282I and S358A
W70Y, S133T and S358A
W70Y, S200N and V124I
W70Y, L282F and V124I

W70Y, L282I and V124I
 W70Y, S133T and V124I
 W70Y, S200N and S133T
 W70Y, L282F and S133T
 W70Y, L282I and S133T
 W70Y, S200N, S133T and L225F
 W70Y, L282F, S133T and L225F
 W70Y, L282I, S133T and L225F
 W70Y, S200N, S133T and S358A
 W70Y, L282F, S133T and S358A
 W70Y, S200N, S133T and V124I
 L225F and I14V
 L225F and I15V
 L225F and S358A
 L225F and V124I
 L225F, S358A and I14V
 L225F, V124I and I15V
 S200N, L225F and I14V
 S200N, L225F and I15V
 S200N, L225F and S358A
 S200N, L225F and V124I
 S200N, L225F, S358A and I14V
 S200N, L225F, V124I and I15V
 S200N, L282I, S133T and L225F
 S200N, L282F, S133T, L225F and S358A

said positions being defined with reference to SEQ ID NO: 2, and wherein the variant
 has one or more altered properties as compared with a reference polypeptide having
 alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2), preferably an
 5 improved property selected from a) to j) as described herein.

The invention further provides a variant polypeptide having alpha-amylase activity,
 wherein the variant has an amino acid sequence which, when aligned with the alpha-
 amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the
 10 following sets of substitutions:

W70Y and S200N
W70Y and L282F
W70Y and L282I
W70Y and L225F
W70Y and I14V
W70Y and I15V
W70Y and S358A
W70Y and V124I
W70Y, S200N and L225F
W70Y, L282F and L225F
W70Y, L282I and L225F
W70Y, S133T and L225F
W70Y, S200N and S358A
W70Y, L282F and S358A
W70Y, L282I and S358A
W70Y, S133T and S358A
W70Y, S200N and V124I
W70Y, L282F and V124I
W70Y, L282I and V124I
W70Y, S133T and V124I
W70Y, S200N and S133T
W70Y, L282F and S133T
W70Y, L282I and S133T
W70Y, S200N, S133T and L225F
W70Y, L282F, S133T and L225F
W70Y, L282I, S133T and L225F
W70Y, S200N, S133T and S358A
W70Y, L282F, S133T and S358A
W70Y, S200N, S133T and V124I

said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has at least one altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2), preferably an improved property selected from a) to j) as described herein.

5

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

L225F and I14V
L225F and I15V
L225F and S358A
L225F and V124I
L225F, S358A and I14V
L225F, V124I and I15V
S200N, L225F and I14V

said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has at least one altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2), preferably an improved property selected from a) to j) as described herein.

10 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

S200N, L225F and I14V
S200N, L225F and I15V
S200N, L225F and S358A
S200N, L225F and V124I
S200N, L225F, S358A and I14V
S200N, L225F, V124I and I15V
S200N, L282I, S133T and L225F
S200N, L282F, S133T, L225F and S358A

15 said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has at least one altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2), preferably an improved property selected from a) to j) as described herein.

20 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the

alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358,

5 said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises the amino acid
10 substitution S200N and at least one further substitution of an amino acid residue corresponding to any of amino acids of 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).
15

In an embodiment the variant polypeptide according to the invention is a variant having alpha-amylase activity, wherein the variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when
20 aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

25 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha amylase activity as set out in SEQ ID NO: 2.

In an embodiment the variant polypeptide according to the invention is a variant having alpha-amylase activity, wherein the variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when
30 aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue

corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha amylase activity as set out in SEQ ID NO: 2 selected
5 from an increased thermostability, an increased sucrose tolerance, an increased an increased Activity at pH4 : Activity at pH5 ratio and an increased Activity at pH7 : Activity at pH5 ratio.

The invention further provides a variant polypeptide having alpha-amylase
10 activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises the amino acid substitution S200N and one or more of I14V, I15V, W70Y, V124I, S133T, S200N, L282I, L282F and S358A, said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a
15 reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a
20 substitution of the amino acid residues corresponding to amino acids 133 and 200, said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase
25 activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises the amino acid substitutions S200N and S133T, said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of
30 SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

W70Y and S200N;
 W70Y, S200N and L225F;
 W70Y, S200N and S358A;
 W70Y, S200N and V124I;
 W70Y, S200N and S133T;
 W70Y, S200N, S133T and L225F;
 W70Y, S200N, S133T and S358A;
 W70Y, S200N, S133T and V124I;
 S200N, L225F and I14V;
 S200N, L225F and I15V;
 S200N, L225F and S358A;
 S200N, L225F and V124I;
 S200N, L225F, S358A and I14V;
 S200N, L225F, V124I and I15V;
 S200N, L282F, S133T and L225F;
 S200N, L282I, S133T and L225F;
 S200N, L282F, S133T, L225F and S358A;
 S200N, L282I, S133T, L225F and S358A;
 S200N, L282F, S133T, L225F and V124I; or
 S200N, L282I, S133T, L225F and V124I,

said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

5 A "substitution" in the context of the present disclosure indicates that a position in the variant which corresponds to one of the positions set out above in SEQ ID NO: 2 comprises an amino acid residue which does not appear at that position in the reference polypeptide (the reference polypeptide may be SEQ ID NO: 2).

Substitutions are also referred herein as Amino acid changes.

10 Preferred substitutions are set out in the Table 1 (with the positions being defined in relation to the sequence set out in SEQ ID NO: 2).

A variant of the invention may be generated using any combination of substitutions set out in Table 1.

Table 1. Preferred substitutions defined in relation to SEQ ID NO: 2

Amino acids are depicted according to the single letter annotation

Variant#	Substitutions *)
V01	W70Y and S200N
V02	W70Y and L282F
V03	W70Y and L282I
V04	W70Y and S133T
V05	W70Y and L225F
V06	W70Y and I14V
V07	W70Y and I15V
V08	W70Y and S358A
V09	W70Y and V124I
V10	W70Y, S200N and L225F
V11	W70Y, L282F and L225F
V12	W70Y, L282I and L225F
V13	W70Y, S133T and L225F
V14	W70Y, S200N and S358A
V15	W70Y, L282F and S358A
V16	W70Y, L282I and S358A
V17	W70Y, S133T and S358A
V18	W70Y, S200N and V124I
V19	W70Y, L282F and V124I
V20	W70Y, L282I and V124I
V21	W70Y, S133T and V124I
V22	W70Y, S200N and S133T
V23	W70Y, L282F and S133T
V24	W70Y, L282I and S133T
V25	W70Y, S200N, S133T and L225F
V26	W70Y, L282F, S133T and L225F
V27	W70Y, L282I, S133T and L225F
V28	W70Y, S200N, S133T and S358A
V29	W70Y, L282F, S133T and S358A
V30	W70Y, S200N, S133T and V124I

V31	W70Y, L282F, S133T and V124I
V32	L225F and I14V
V33	L225F and I15V
V34	L225F and S358A
V35	L225F and V124I
V36	L225F, S358A and I14V
V37	L225F, V124I and I15V
V38	S200N, L225F and I14V
V39	S200N, L225F and I15V
V40	S200N, L225F and S358A
V41	S200N, L225F and V124I
V42	S200N, L225F, S358A and I14V
V43	S200N, L225F, V124I and I15V
V44	S200N, L282F, S133T and L225F
V45	S200N, L282I, S133T and L225F
V46	S200N, L282F, S133T, L225F and S358A
V47	S200N, L282I, S133T, L225F and S358A
V48	S200N, L282F, S133T, L225F and V124I
V49	S200N, L282I, S133T, L225F and V124I
V50	Reference alpha-amylase having an amino acid sequence as set out in SEQ ID NO 2
V51	S200N
V52	W70Y
V53	L282F
V54	L282I
V55	S133T
V56	L225F
V57	I14V
V58	I15V
V59	S358A
V60	V124I

*) W70Y and S200N means 2 changes: the amino acid at position 70 and the amino acid at position 200 are changed. The W (Tryptophan) at position 70 is changed into a Y (Tyrosine) and that S (Serine) at position 200 is changed into a N (Asparagine), etc.

- 5 A variant of the invention was generated using any combination of substitutions set out in Table 2. Under the same conditions a reference alpha-amylase polypeptide having an amino acid sequence as set out in SEQ ID NO 2 was generated.

Table 2. Preferred substitutions defined in relation to SEQ ID NO: 2

- 10 Amino acids are depicted according to the single letter annotation

Variant#	Substitutions *)
V01	W70Y and S200N
V02	W70Y and L282F
V03	W70Y and L282I
V05	W70Y and L225F
V06	W70Y and I14V
V07	W70Y and I15V
V08	W70Y and S358A
V09	W70Y and V124I
V10	W70Y, S200N and L225F
V11	W70Y, L282F and L225F
V12	W70Y, L282I and L225F
V13	W70Y, S133T and L225F
V14	W70Y, S200N and S358A
V15	W70Y, L282F and S358A
V16	W70Y, L282I and S358A
V17	W70Y, S133T and S358A
V18	W70Y, S200N and V124I
V19	W70Y, L282F and V124I
V20	W70Y, L282I and V124I
V21	W70Y, S133T and V124I
V22	W70Y, S200N and S133T
V23	W70Y, L282F and S133T

V24	W70Y, L282I and S133T
V25	W70Y, S200N, S133T and L225F
V26	W70Y, L282F, S133T and L225F
V27	W70Y, L282I, S133T and L225F
V28	W70Y, S200N, S133T and S358A
V29	W70Y, L282F, S133T and S358A
V30	W70Y, S200N, S133T and V124I
V32	L225F and I14V
V33	L225F and I15V
V34	L225F and S358A
V35	L225F and V124I
V36	L225F, S358A and I14V
V37	L225F, V124I and I15V
V38	S200N, L225F and I14V
V39	S200N, L225F and I15V
V40	S200N, L225F and S358A
V41	S200N, L225F and V124I
V42	S200N, L225F, S358A and I14V
V43	S200N, L225F, V124I and I15V
V45	S200N, L282I, S133T and L225F
V46	S200N, L282F, S133T, L225F and S358A
V51	S200N
V52	W70Y
V53	L282F
V55	S133T
V56	L225F
V57	I14V
V59	S358A
V60	V124I

*) W70Y and S200N means 2 changes: the amino acid at position 70 and the amino acid at position 200 are changed. The W (Tryptophan) at position 70 is changed into a Y (Tyrosine) and that S (Serine) at position 200 is changed into a N (Asparagine), etc.

A variant alpha-amylase of the invention may also comprise additional modifications in comparison to the parent polypeptide at positions other than those specified above, for example, one or more additional substitutions, additions or deletions. A variant of the invention may comprise a combination of different types of modification of this sort. A variant may comprise one, two, three, four, least 5, at least 10, at least 15, at least 20, at least 25, at least 30 or more such modifications (which may all be of the same type or may be different types of modification). Typically, the additional modifications may be substitutions.

The invention thus also provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue corresponding to any of amino acids

14, 15, 124, 133, 225, 200, 282, 358,

said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant polypeptide comprises at least one additional substitution other than those defined herein.

The invention thus also provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 and at least one further substitution of an amino acid residue corresponding to any of amino acids

14, 15, 124, 133, 200, 282, 358,

said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant polypeptide comprises at least one additional substitution other than those defined herein.

The invention thus also provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 and at least one

further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358,

5 said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant polypeptide comprises at least one additional substitution other than those defined herein.

10 In an aspect the alpha-amylase variant of the invention (for example a variant having one or more substitution as set out in Table 1) comprises an amino acid sequence having at least 70% identity to the parent polypeptide, such as the alpha-amylase of SEQ ID NO: 2, in an aspect at least 80% identity, in an aspect at least 85% identity, in an aspect at least 90% identity, in an aspect at least 95% identity, in an aspect at least 96% identity, in an aspect at least 97% identity, in an aspect at least 98% identity, in an aspect at least 99% identity to the parent polypeptide. Such a variant will
15 typically have one or more substitutions or sets of substitutions as set out in Table 1.

In an aspect the variant will have one or more substitutions or sets of substitutions as set out in Table 2.

The terms alpha-amylase variant of the invention, variant polypeptide having alpha amylase activity and alpha-amylase polypeptide variant are used interchangeably herein.

20 The terms reference polypeptide and parent polypeptide are used interchangeably herein.

In an aspect the variant polypeptide having alpha-amylase activity of the invention (for example a variant having one or more substitution as set out in Table 1) comprises an amino acid sequence having at least 70% identity to the parent polypeptide, such as the
25 alpha-amylase of SEQ ID NO: 2, in an aspect at least 75% identity, in an aspect at least 80% identity, in an aspect at least 85% identity, in an aspect at least 90% identity, in an aspect at least 95% identity, in an aspect at least 96% identity, in an aspect at least 97% identity, in an aspect at least 98% identity, in an aspect at least 99% identity to the parent polypeptide.

30 In an aspect the variant polypeptide having alpha-amylase activity of the invention (for example a variant having one or more substitution as set out in Table 2) comprises an amino acid sequence having at least 70% identity to the parent polypeptide, such as the alpha-amylase of SEQ ID NO: 2, in an aspect at least 75% identity, in an aspect at least 80% identity, in an aspect at least 85% identity, in an aspect at least 90% identity, in an

aspect at least 95% identity, in an aspect at least 96% identity, in an aspect at least 97% identity, in an aspect at least 98% identity, in an aspect at least 99% identity to the parent polypeptide.

5 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 and at least one further
10 substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates one or more altered properties as compared with a reference polypeptide having alpha-amylase activity, with the proviso that the variant is not the polypeptide encoded by the wildtype DNA as set out in SEQ ID NO: 1.

15 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 and at least one further
20 substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates one or more altered properties as compared with a reference polypeptide having alpha-amylase activity, with the proviso that the variant is not the polypeptide encoded by the wildtype DNA as set out in SEQ ID NO: 1.

25 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 and at least one further
30 substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates one or more altered properties as compared with a reference polypeptide having alpha-amylase activity, with the proviso that the variant is not the polypeptide encoded by the wildtype DNA as set out in SEQ ID NO: 1.

In an aspect of the invention, the variant polypeptide is a variant which is not the polypeptide encoded by the wildtype DNA as set out in SEQ ID NO: 1.

Optionally, a variant polypeptide is not a naturally-occurring polypeptide.

5 In an embodiment the present invention provides a variant polypeptide having alpha-amylase activity, wherein said variant has an amino acid sequence having at least 70% identity to the amino acid sequence as set out in SEQ ID NO: 2 which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid W70Y
10 and at least one further substitution of an amino acid residue corresponding to any of amino acids

14, 15, 124, 133, 225, 200, 282, 358,

said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has one or more altered properties as compared with a reference polypeptide having
15 alpha-amylase activity as set out in SEQ ID NO: 2.

In an embodiment the present invention provides a variant polypeptide having alpha-amylase activity, wherein said variant has an amino acid sequence having at least 70% identity to the amino acid sequence as set out in SEQ ID NO: 2 which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2,
20 comprises a substitution of an amino acid residue corresponding to amino acid L225F and at least one further substitution of an amino acid residue corresponding to any of amino acids

14, 15, 124, 133, 200, 282, 358,

said positions being defined with reference to SEQ ID NO: 2, and wherein the variant has one or more altered properties as compared with a reference polypeptide having
25 alpha-amylase activity as set out in SEQ ID NO: 2.

In an embodiment the present invention provides a variant polypeptide having alpha-amylase activity, wherein said variant has an amino acid sequence having at least 70% identity to the amino acid sequence as set out in SEQ ID NO: 2 which, when
30 aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid S200N and at least one further substitution of an amino acid residue corresponding to any of

amino acids

14, 15, 70, 124, 133, 225, 282, 358,

said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity as set out in SEQ ID NO: 2.

In an aspect the alpha-amylase variant of the invention comprises an amino acid sequence having at least one of Asp at position 151, Ala at position 264, Thr at position 335 and Asn at position 456, said positions being defined with reference to SEQ ID NO: 2. In an aspect the alpha-amylase variant of the invention comprises an amino acid sequence having all of Asp at position 151, Ala at position 264, Thr at position 335 and Asn at position 456, said positions being defined with reference to SEQ ID NO: 2.

A variant of the invention will typically retain alpha-amylase activity.

The alpha-amylase variant according to the invention and the parent polypeptide herein are a starch degrading enzymes. The alpha-amylase variant according to the invention and the parent polypeptide herein have alpha-amylase activity. Alpha-amylase activity can suitably be determined using the Ceralpha® procedure, which is recommended by the American Association of Cereal Chemists (AACC).

Enzymatic activity of an alpha-amylase variant and of a parent polypeptide may be expressed as NBAU. NBAU activity can suitably be determined using the NBAU assay as described herein.

Preferably, a variant of the invention will typically exhibit improved properties in comparison with the reference alpha-amylase polypeptide from which it is derived. Such an improved property will typically be one which is relevant if the variant were to be used as set out herein, for example in a method for preparing a baked product.

A variant which exhibits a property which is improved in relation to the reference alpha-amylase is one which demonstrates a measurable reduction or increase in the relevant property, typically such that the variant is more suited to use as set out herein, for example in a method for the production of a baked product.

The property may thus be decreased by at least 10%, at least 20%, at least 30%, at least 40% at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99%. Alternatively, the property may be increased by at least

10%, at least 25%, at least 50%, at least 100%, at least, 200%, at least 500% or at least 1000%. The percentage decrease or increase in this context represents the percentage decrease or increase in comparison to the reference alpha-amylase polypeptide. It is well known to the skilled person how such percentage changes may be measured – it is
5 a comparison of the activity of the reference alpha-amylase and the variant alpha-amylase.

The variants described herein are collectively comprised in the terms “a polypeptide according to the invention” or “a variant according to the invention” or “a variant polypeptide according to the invention”.

10

The one or more altered properties of alpha-amylase variant according to the invention may include without limitation one or more of

- a) an increased thermostability;
- b) an increased sucrose tolerance;
- 15 c) an increased thermostability in the presence of sucrose;
- d) an increased Activity at pH4 : Activity at pH5 ratio;
- e) an increased Activity at pH7 : Activity at pH5 ratio; and
an increased specific activity,

as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.
20 The improved property may include

- g) an increased ratio of the activity at a temperature of 50 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.
25
- h) The improved property may include an increased ratio of the activity at a temperature of 60 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.
30
- i) an increased ratio of the activity at a temperature of 60 degrees Celsius versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

j) The improved property may include an increased ratio of the activity at a temperature of 80 degrees Celsius versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased thermostability as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased sucrose tolerance as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased - Activity at pH4 : Activity at pH5 ratio as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased Activity at pH7 : Activity at pH5 ratio as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased specific activity at pH 4 as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased specific activity at pH 5 as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an

increased specific activity at pH 7 as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of
5 an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an
10 increased ratio of the activity at a temperature of 50 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

The invention further provides a variant polypeptide having alpha-amylase activity,
15 wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions
20 being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased ratio of the activity at a temperature of 60 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of
25 an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions
30 being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased ratio of the activity at a temperature of 60 degrees Celsius versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having

alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of
5 an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an
10 increased thermostability as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of
15 an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an
20 increased sucrose tolerance as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of
25 an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an
increased Activity at pH4 : Activity at pH5 ratio as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

30 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue

corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased Activity at pH7 : Activity at pH5 ratio as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

5 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue
10 corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased specific activity at pH 4 as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

 The invention further provides a variant polypeptide having alpha-amylase activity,
15 wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions
20 being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased specific activity at pH 5 as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of
25 an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an
30 increased specific activity at pH 7 as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of

an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased ratio of the activity at a temperature of 50 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased ratio of the activity at a temperature of 60 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased ratio of the activity at a temperature of 60 degrees Celsius versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably

comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased thermostability as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased sucrose tolerance as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased Activity at pH4 : Activity at pH5 ratio as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased Activity at pH7 : Activity at pH5 ratio as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased specific activity at pH 4 as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased specific activity at pH 5 as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased specific activity at pH 7 as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an

increased ratio of the activity at a temperature of 50 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

5 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue
10 corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased ratio of the activity at a temperature of 60 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2)
15 measured under the same conditions.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue
20 corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2, and wherein the variant demonstrates an increased ratio of the activity at a temperature of 60 degrees Celsius versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having
25 alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions. In an aspect the alpha-amylase variant according to the invention has two altered properties, in an aspect three altered properties selected from a) to f) above as compared to a reference polypeptide having alpha-amylase activity. In a preferred aspect the reference polypeptide having alpha-amylase activity is the
30 polypeptide as set out in SEQ ID NO: 2.

In an aspect the alpha-amylase variant according to the invention has two altered properties, in an aspect three altered properties selected from a) to g) above as compared to a reference polypeptide having alpha-amylase activity. In a preferred

aspect the reference polypeptide having alpha-amylase activity is the polypeptide as set out in SEQ ID NO: 2.

In an aspect the alpha-amylase variant according to the invention has two altered properties, in an aspect three altered properties selected from a) to j) described herein as compared to a reference polypeptide having alpha-amylase activity. In a preferred aspect the reference polypeptide having alpha-amylase activity is the polypeptide as set out in SEQ ID NO: 2.

In an aspect the alpha-amylase variant according to the invention has both an increased thermostability and an increased specific activity at pH 4 as compared to a reference polypeptide having alpha-amylase activity, wherein the reference polypeptide is the alpha-amylase as set in SEQ ID NO:2.

In an aspect the alpha-amylase variant according to the invention has both an increased thermostability and an increased specific activity at pH 5 as compared to a reference polypeptide having alpha-amylase activity, wherein the reference polypeptide is the alpha-amylase as set in SEQ ID NO:2.

In an aspect the alpha-amylase variant according to the invention has both an increased thermostability and an increased specific activity at pH 7 as compared to a reference polypeptide having alpha-amylase activity, wherein the reference polypeptide is the alpha-amylase as set in SEQ ID NO:2.

In an aspect the alpha-amylase variant according to the invention has both an increased thermostability and an increased sucrose tolerance as compared to a reference polypeptide having alpha-amylase activity, wherein the reference polypeptide is the alpha-amylase as set in SEQ ID NO:2.

In an aspect the alpha-amylase variant according to the invention has both an increased thermostability and an increased sucrose tolerance at pH 7 as compared to a reference polypeptide having alpha-amylase activity, wherein the reference polypeptide is the alpha-amylase as set in SEQ ID NO:2.

In an aspect the alpha-amylase variant according to the invention has both an increased thermostability at pH 7 and an increased sucrose tolerance at pH 7 as compared to a reference polypeptide having alpha-amylase activity, wherein the reference polypeptide is the alpha-amylase as set in SEQ ID NO:2.

In an aspect the alpha-amylase variant according to the invention has an increased sucrose tolerance and an increased thermostability; as compared to a

reference polypeptide having alpha-amylase activity, wherein the reference polypeptide is the alpha-amylase as set in SEQ ID NO:2.

In an aspect the alpha-amylase variant according to the invention has an increased sucrose tolerance at pH 7, an increased Activity pH7 : Activity at pH5 ratio and an increased thermostability at pH 7 as compared to a reference polypeptide having alpha-amylase activity, wherein the reference polypeptide is the alpha-amylase as set in SEQ ID NO:2.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased thermostability compared with the reference polypeptide as set out in SEQ ID NO: 2, while the specific activity at pH 4 of the variant has reduced by at most 20% as compared with the reference polypeptide (such as the polypeptide of SEQ ID NO: 2).

In an aspect of this embodiment the specific activity at pH 4 of the variant has reduced by at most 30%, in an aspect by at most 40%, in an aspect by at most 50% as compared to the reference polypeptide. In a further aspect of this embodiment the specific activity at pH 4 of the variant is at least the same or has increased compared to the reference polypeptide.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased thermostability compared with the reference polypeptide as set out in SEQ ID NO: 2, while the specific activity at pH 5 of the variant has reduced by at most 20% as compared with the reference polypeptide (such as the polypeptide of SEQ ID NO: 2).

5 In an aspect of this embodiment the specific activity at pH 5 of the variant has reduced by at most 30%, in an aspect by at most 40%, in an aspect by at most 50% as compared to the reference polypeptide. In a further aspect of this embodiment the specific activity at pH 5 of the variant is at least the same or has increased compared to the reference polypeptide.

10 In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y)
15 and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased thermostability compared with the reference polypeptide as set out in SEQ ID NO: 2, while the specific activity at pH 7 of the variant
20 has reduced by at most 20% as compared with the reference polypeptide (such as the polypeptide of SEQ ID NO: 2).

In an aspect of this embodiment the specific activity at pH 7 of the variant has reduced by at most 30%, in an aspect by at most 40%, in an aspect by at most 50% as compared to the reference polypeptide. In a further aspect of this embodiment the specific activity
25 at pH 7 of the variant is at least the same or has increased compared to the reference polypeptide.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the
30 sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased thermostability compared with the reference polypeptide as set out in SEQ ID NO: 2, while the specific activity at pH 4 of the variant has reduced by at most 20% as compared with the reference polypeptide (such as the polypeptide of SEQ ID NO: 2).

5 In an aspect of this embodiment the specific activity at pH 4 of the variant has reduced by at most 30%, in an aspect by at most 40%, in an aspect by at most 50% as compared to the reference polypeptide. In a further aspect of this embodiment the specific activity at pH 4 of the variant is at least the same or has increased compared to the reference polypeptide.

10 In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution
15 L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased thermostability compared with the reference polypeptide as set out in SEQ ID NO: 2, while the specific activity at pH 5 of the variant
20 has reduced by at most 20% as compared with the reference polypeptide (such as the polypeptide of SEQ ID NO: 2).

In an aspect of this embodiment the specific activity at pH 5 of the variant has reduced by at most 30%, in an aspect by at most 40%, in an aspect by at most 50% as compared to the reference polypeptide. In a further aspect of this embodiment the specific activity
25 at pH 5 of the variant is at least the same or has increased compared to the reference polypeptide.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the
30 sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased thermostability compared with the reference polypeptide as set out in SEQ ID NO: 2, while the specific activity at pH 7 of the variant has reduced by at most 20% as compared with the reference polypeptide (such as the polypeptide of SEQ ID NO: 2).

5 In an aspect of this embodiment the specific activity at pH 7 of the variant has reduced by at most 30%, in an aspect by at most 40%, in an aspect by at most 50% as compared to the reference polypeptide. In a further aspect of this embodiment the specific activity at pH 7 of the variant is at least the same or has increased compared to the reference polypeptide.

10 In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution
15 S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased thermostability compared with the reference polypeptide as set out in SEQ ID NO: 2, while the specific activity at pH 4 of the variant
20 has reduced by at most 20% as compared with the reference polypeptide (such as the polypeptide of SEQ ID NO: 2).

In an aspect of this embodiment the specific activity at pH 4 of the variant has reduced by at most 30%, in an aspect by at most 40%, in an aspect by at most 50% as compared to the reference polypeptide. In a further aspect of this embodiment the specific activity
25 at pH 4 of the variant is at least the same or has increased compared to the reference polypeptide.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the
30 sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased thermostability compared with the reference polypeptide as set out in SEQ ID NO: 2, while the specific activity at pH 5 of the variant has reduced by at most 20% as compared with the reference polypeptide (such as the polypeptide of SEQ ID NO: 2).

5 In an aspect of this embodiment the specific activity at pH 5 of the variant has reduced by at most 30%, in an aspect by at most 40%, in an aspect by at most 50% as compared to the reference polypeptide. In a further aspect of this embodiment the specific activity at pH 5 of the variant is at least the same or has increased compared to the reference polypeptide.

10 In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution
15 S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased thermostability compared with the reference polypeptide as set out in SEQ ID NO: 2, while the specific activity at pH 7 of the variant
20 has reduced by at most 20% as compared with the reference polypeptide (such as the polypeptide of SEQ ID NO: 2).

In an aspect of this embodiment the specific activity at pH 7 of the variant has reduced by at most 30%, in an aspect by at most 40%, in an aspect by at most 50% as compared to the reference polypeptide. In a further aspect of this embodiment the specific activity
25 at pH 7 of the variant is at least the same or has increased compared to the reference polypeptide.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the
30 polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of

amino acids 15, 124, 200, 225, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased sucrose tolerance at pH 7, an increased Activity pH7 : Activity at pH5 ratio and an increased thermostability at pH 7 compared
5 with the reference polypeptide as set out in SEQ ID NO: 2.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the
10 sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 15, 124, 200, 225, 358, said positions being defined with reference to SEQ ID NO: 2;

15 and wherein the variant has an increased sucrose tolerance at pH 7 and an increased thermostability at pH 7 compared with the reference polypeptide as set out in SEQ ID NO: 2.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the
20 sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of
25 amino acids 15, 124, 200, 225, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased sucrose tolerance compared with the reference polypeptide as set out in SEQ ID NO: 2.

30 In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y)

and at least one further substitution of an amino acid residue corresponding to amino acid 225, said position being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased sucrose tolerance at pH7 compared with the reference polypeptide as set out in SEQ ID NO: 2.

5

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue
10 corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased thermostability compared with the reference
15 polypeptide as set out in SEQ ID NO: 2.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the
20 sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to amino acid 200, said position being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased thermostability compared with the reference
25 polypeptide as set out in SEQ ID NO: 2.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the
30 sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 200, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased Activity pH7 : Activity at pH5 ratio compared with the reference polypeptide as set out in SEQ ID NO: 2.

5 In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue
10 any of amino acids 14, 15, 124, 358, said positions being defined with reference to SEQ ID NO: 2;
and wherein the variant has an increased sucrose tolerance compared with the reference polypeptide as set out in SEQ ID NO: 2.

15 In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution
20 W70Y) and at least one further substitution of an amino acid residue corresponding to amino acid 358, said position being defined with reference to SEQ ID NO: 2;
and wherein the variant has an increased sucrose tolerance at pH5 compared with the reference polypeptide as set out in SEQ ID NO: 2.

25 In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution
30 L225F) and at least one further substitution of an amino acid residue corresponding to amino acid 358, said position being defined with reference to SEQ ID NO: 2;
and wherein the variant has an increased Activity pH7 : Activity at pH5 ratio compared with the reference polypeptide as set out in SEQ ID NO: 2.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to amino acid 358, said position being defined with reference to SEQ ID NO: 2; and wherein the variant has an increased Activity pH4 : Activity at pH5 ratio compared with the reference polypeptide as set out in SEQ ID NO: 2.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to amino acid 358, said position being defined with reference to SEQ ID NO: 2; and wherein the variant has an increased Activity at 50°C or higher : Activity at 37°C ratio compared with the reference polypeptide as set out in SEQ ID NO: 2.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to amino acid 358 said position being defined with reference to SEQ ID NO: 2; and wherein the variant has an increased Activity at 60°C : Activity at 37°C ratio compared with the reference polypeptide as set out in SEQ ID NO: 2.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution

W70Y) and at least one further substitution of an amino acid residue corresponding to amino acid 358 said position being defined with reference to SEQ ID NO: 2;

and wherein the variant has an increased Activity at 80°C : Activity at 37°C ratio compared with the reference polypeptide as set out in SEQ ID NO: 2.

- 5 In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution
- 10 L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 70, 133, 200, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2;
- and wherein the variant has an altered property compared with the reference polypeptide as set out in SEQ ID NO: 2. In an aspect the altered property is one or more of an
- 15 increased sucrose tolerance; an increased thermostability; and an increased thermostability in the presence of sucrose.

- In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence having at least 70% identity with the
- 20 polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 70, 133, 200, 225, 282, 358, said positions being defined with
- 25 reference to SEQ ID NO: 2;
- and wherein the variant has an altered property compared with the reference polypeptide as set out in SEQ ID NO: 2. In an aspect the altered property is one or more of an increased sucrose tolerance; an increased thermostability; an increased thermostability in the presence of sucrose; Activity at 60°C : Activity at 37°C ratio and an Activity at
- 30 80°C : Activity at 37°C ratio.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

W70Y, L282F and L225F

W70Y, L282I and L225F

W70Y, L282I, S133T and L225F

S200N, L282I, S133T and L225F

S200N, L282F, S133T, L225F and S358A

- 5 said positions being defined with reference to SEQ ID NO: 2,
and wherein the variant has an altered property compared with the reference polypeptide as set out in SEQ ID NO: 2. In an aspect the altered property is one or more of an increased sucrose tolerance; an increased thermostability; and an increased thermostability in the presence of sucrose.

10

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

W70Y, L282F and L225F

W70Y, L282I and L225F

W70Y, L282I, S133T and L225F

S200N, L282I, S133T and L225F

S200N, L282F, S133T, L225F and S358A

- 15 said positions being defined with reference to SEQ ID NO: 2,
and wherein the variant has an altered property compared with the reference polypeptide as set out in SEQ ID NO: 2. In an aspect the altered property is an increased sucrose tolerance and an increased thermostability.

- 20 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

W70Y and S200N

W70Y and L225F
W70Y and I15V
W70Y and S358A
W70Y and V124I
W70Y, S200N and L225F
W70Y, L282F and L225F
W70Y, L282I and L225F
W70Y, S133T and L225F
W70Y, S200N and S358A
W70Y, L282F and S358A
W70Y, S133T and S358A
W70Y, S200N and S133T
W70Y, S200N, S133T and L225F
W70Y, L282F, S133T and L225F
W70Y, L282I, S133T and L225F
W70Y, S200N, S133T and S358A
W70Y, L282F, S133T and S358A
W70Y, S200N, S133T and V124I
L225F and I14V
L225F and I15V
L225F and S358A
L225F and V124I
L225F, S358A and I14V
L225F, V124I and I15V
S200N, L225F and I14V
S200N, L225F and I15V
S200N, L225F and S358A
S200N, L225F and V124I
S200N, L225F, S358A and I14V
S200N, L225F, V124I and I15V
S200N, L282I, S133T and L225F
S200N, L282F, S133T, L225F and S358A

said positions being defined with reference to SEQ ID NO: 2,

and wherein the variant has an increased sucrose tolerance compared with the reference polypeptide as set out in SEQ ID NO: 2.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

W70Y and S200N
W70Y and L225F
W70Y and I15V
W70Y and S358A
W70Y and V124I
W70Y, S200N and L225F
W70Y, L282F and L225F
W70Y, L282I and L225F
W70Y, S133T and L225F
W70Y, S200N and S358A
W70Y, L282F and S358A
W70Y, S133T and S358A
W70Y, S200N and S133T
W70Y, S200N, S133T and L225F
W70Y, L282F, S133T and L225F
W70Y, L282I, S133T and L225F
W70Y, S200N, S133T and S358A
W70Y, L282F, S133T and S358A
W70Y, S200N, S133T and V124I

said positions being defined with reference to SEQ ID NO: 2,

and wherein the variant has an increased sucrose tolerance compared with the reference polypeptide as set out in SEQ ID NO: 2.

10 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

W70Y and L225F

W70Y, S200N and L225F
W70Y, L282F and L225F
W70Y, L282I and L225F
W70Y, S133T and L225F
W70Y, S200N, S133T and L225F
W70Y, L282F, S133T and L225F
W70Y, L282I, S133T and L225F
L225F and I14V
L225F and I15V
L225F and S358A
L225F and V124I
L225F, S358A and I14V
L225F, V124I and I15V
S200N, L225F and I14V
S200N, L225F and I15V
S200N, L225F and S358A
S200N, L225F and V124I
S200N, L225F, S358A and I14V
S200N, L225F, V124I and I15V
S200N, L282I, S133T and L225F
S200N, L282F, S133T, L225F and S358A

said positions being defined with reference to SEQ ID NO: 2,

and wherein the variant has an increased sucrose tolerance compared with the reference polypeptide as set out in SEQ ID NO: 2.

The invention further provides a variant polypeptide having alpha-amylase activity,
5 wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

W70Y and S200N
W70Y and L282F
W70Y and L282I
W70Y and I14V
W70Y and I15V

W70Y and S358A
W70Y and V124I
W70Y, S200N and L225F
W70Y, L282F and L225F
W70Y, L282I and L225F
W70Y, S133T and L225F
W70Y, S200N and S358A
W70Y, L282F and S358A
W70Y, L282I and S358A
W70Y, S133T and S358A
W70Y, S200N and V124I
W70Y, L282F and V124I
W70Y, L282I and V124I
W70Y, S133T and V124I
W70Y, S200N and S133T
W70Y, L282F and S133T
W70Y, S200N, S133T and L225F
W70Y, L282F, S133T and L225F
W70Y, L282I, S133T and L225F
W70Y, S200N, S133T and S358A
W70Y, L282F, S133T and S358A
W70Y, S200N, S133T and V124I
S200N, L225F and I14V
S200N, L225F and V124I
S200N, L282I, S133T and L225F
S200N, L282F, S133T, L225F and S358A

said positions being defined with reference to SEQ ID NO: 2,
and wherein the variant has an increased thermostability compared with the reference
polypeptide as set out in SEQ ID NO: 2.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

W70Y and S200N
W70Y and L282F
W70Y and L282I
W70Y and I14V
W70Y and I15V
W70Y and S358A
W70Y and V124I
W70Y, S200N and L225F
W70Y, L282F and L225F
W70Y, L282I and L225F
W70Y, S133T and L225F
W70Y, S200N and S358A
W70Y, L282F and S358A
W70Y, L282I and S358A
W70Y, S133T and S358A
W70Y, S200N and V124I
W70Y, L282F and V124I
W70Y, L282I and V124I
W70Y, S133T and V124I
W70Y, S200N and S133T
W70Y, L282F and S133T
W70Y, S200N, S133T and L225F
W70Y, L282F, S133T and L225F
W70Y, L282I, S133T and L225F
W70Y, S200N, S133T and S358A
W70Y, L282F, S133T and S358A
W70Y, S200N, S133T and V124I

- 5 said positions being defined with reference to SEQ ID NO: 2,
and wherein the variant has an increased thermostability compared with the reference polypeptide as set out in SEQ ID NO: 2.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

W70Y and S200N
W70Y, S200N and L225F
W70Y, S200N and S358A
W70Y, S200N and V124I
W70Y, S200N and S133T
W70Y, S200N, S133T and L225F
W70Y, S200N, S133T and S358A
W70Y, S200N, S133T and V124I
S200N, L225F and I14V
S200N, L225F and V124I
S200N, L282I, S133T and L225F
S200N, L282F, S133T, L225F and S358A

- 5 said positions being defined with reference to SEQ ID NO: 2,
and wherein the variant has an increased thermostability compared with the reference polypeptide as set out in SEQ ID NO: 2.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-
10 amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

W70Y and S200N
W70Y and L225F
W70Y and I15V
W70Y and S358A
W70Y and V124I
W70Y, S200N and L225F
W70Y, L282F and L225F
W70Y, L282I and L225F
W70Y, S133T and L225F
W70Y, S200N and S358A

W70Y, L282F and S358A
 W70Y, L282I and S358A
 W70Y, S133T and S358A
 W70Y, S133T and V124I
 W70Y, S200N and S133T
 W70Y, S200N, S133T and L225F
 W70Y, L282F, S133T and L225F
 W70Y, L282I, S133T and L225F
 W70Y, S200N, S133T and S358A
 W70Y, L282F, S133T and S358A
 W70Y, S200N, S133T and V124I
 S200N, L225F and I14V
 S200N, L225F and S358A
 S200N, L225F and V124I
 S200N, L225F, S358A and I14V
 S200N, L282I, S133T and L225F
 S200N, L282F, S133T, L225F and S358A

said positions being defined with reference to SEQ ID NO: 2,

and wherein the variant has an increased sucrose tolerance at pH7 and an increased thermostability at pH7 compared with the reference polypeptide as set out in SEQ ID NO: 2.

- 5 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

W70Y and S200N
 W70Y and L225F
 W70Y and I15V
 W70Y and S358A
 W70Y and V124I
 W70Y, S200N and L225F
 W70Y, L282F and L225F
 W70Y, L282I and L225F

W70Y, S133T and L225F
 W70Y, S200N and S358A
 W70Y, L282F and S358A
 W70Y, L282I and S358A
 W70Y, S133T and S358A
 W70Y, S133T and V124I
 W70Y, S200N and S133T
 W70Y, S200N, S133T and L225F
 W70Y, L282F, S133T and L225F
 W70Y, L282I, S133T and L225F
 W70Y, S200N, S133T and S358A
 W70Y, L282F, S133T and S358A
 W70Y, S200N, S133T and V124I

said positions being defined with reference to SEQ ID NO: 2,

and wherein the variant has an increased sucrose tolerance at pH7 and an increased thermostability at pH7 compared with the reference polypeptide as set out in SEQ ID NO: 2.

- 5 The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

S200N, L225F and I14V
 S200N, L225F and S358A
 S200N, L225F and V124I
 S200N, L225F, S358A and I14V
 S200N, L282I, S133T and L225F
 S200N, L282F, S133T, L225F and S358A

said positions being defined with reference to SEQ ID NO: 2,

- 10 and wherein the variant has an increased sucrose tolerance at pH7 and an increased thermostability at pH7 compared with the reference polypeptide as set out in SEQ ID NO: 2.

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-

amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

W70Y and S200N
W70Y and L225F
W70Y and I15V
W70Y and S358A
W70Y and V124I
W70Y, S200N and L225F
W70Y, L282F and L225F
W70Y, L282I and L225F
W70Y, S133T and L225F
W70Y, S200N and S358A
W70Y, L282F and S358A
W70Y, L282I and S358A
W70Y, S133T and S358A
W70Y, S133T and V124I
W70Y, S200N and S133T
W70Y, S200N, S133T and L225F
W70Y, L282F, S133T and L225F
W70Y, L282I, S133T and L225F
W70Y, S200N, S133T and S358A
W70Y, L282F, S133T and S358A
W70Y, S200N, S133T and V124I
S200N, L282I, S133T and L225F
S200N, L282F, S133T, L225F and S358A

said positions being defined with reference to SEQ ID NO: 2,

and wherein the variant has an increased sucrose tolerance at pH7, an increased
5 thermostability at pH7 and an increased Activity at pH7 : Activity at pH5 ratio compared
with the reference polypeptide as set out in SEQ ID NO: 2.

The invention further provides a variant polypeptide having alpha-amylase activity,
wherein the variant has an amino acid sequence which, when aligned with the alpha-
10 amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the
following sets of substitutions:

W70Y and S200N
 W70Y and L225F
 W70Y and I15V
 W70Y and S358A
 W70Y and V124I
 W70Y, S200N and L225F
 W70Y, L282F and L225F
 W70Y, L282I and L225F
 W70Y, S133T and L225F
 W70Y, S200N and S358A
 W70Y, L282F and S358A
 W70Y, L282I and S358A
 W70Y, S133T and S358A
 W70Y, S133T and V124I
 W70Y, S200N and S133T
 W70Y, S200N, S133T and L225F
 W70Y, L282F, S133T and L225F
 W70Y, L282I, S133T and L225F
 W70Y, S200N, S133T and S358A
 W70Y, L282F, S133T and S358A
 W70Y, S200N, S133T and V124I

said positions being defined with reference to SEQ ID NO: 2,

and wherein the variant has an increased sucrose tolerance at pH7, an increased thermostability at pH7 and an increased Activity at pH7 : Activity at pH5 ratio compared with the reference polypeptide as set out in SEQ ID NO: 2.

5

The invention further provides a variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises one of the following sets of substitutions:

S200N, L282I, S133T and L225F

S200N, L282F, S133T, L225F and S358A

10 said positions being defined with reference to SEQ ID NO: 2,

and wherein the variant has an increased sucrose tolerance at pH7, an increased thermostability at pH7 and an increased Activity at pH7 : Activity at pH5 ratio compared with the reference polypeptide as set out in SEQ ID NO: 2.

5 As used herein, the terms "variant", "derivative", "mutant" or "homologue" can be used interchangeably. They can refer to either polypeptides or nucleic acids. Variants include substitutions, insertions, deletions, truncations, transversions, and/or inversions, at one or more locations relative to a reference sequence. Variants can be made for example by site-saturation mutagenesis, scanning mutagenesis, insertional mutagenesis,
10 random mutagenesis, site-directed mutagenesis, and directed-evolution, as well as various other recombination approaches known to a skilled person in the art.

The term 'polypeptide' includes proteins. In one embodiment, the polypeptides according to the invention comprise only conventional or natural amino acids. In the description and claims of the present invention, the single letter code for amino acids is
15 used, where A stands for Alanine, C for Cysteine, D for Aspartic acid, E for Glutamic acid, F for Phenylalanine, G for Glycine, H for Histidine, I for Isoleucine, K for Lysine, L for Leucine, M for Methionine, N for Asparagine, P for Proline, Q for Glutamine, R for Arginine, S for Serine, T for Threonine, V for Valine, W for Tryptophan, Y for Tyrosine. Such single letter codes are commonly known in the art, see e.g. Sambrook, et
20 al. (Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

The terms "peptide" and "oligopeptide" are considered synonymous (as is commonly recognized) and each term can be used interchangeably as the context requires to indicate a chain of at least two amino acids coupled by peptidyl linkages. The
25 word "polypeptide" is used herein for chains containing more than about seven amino acid residues. All oligopeptide and polypeptide formulas or sequences herein are written from left to right and in the direction from amino terminus to carboxy terminus. The one-letter code of amino acids used herein is commonly known in the art and can be found in Sambrook, et al. (Molecular Cloning: A Laboratory Manual, 2nded. Cold Spring Harbor
30 Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

A polypeptide of the invention may be in isolated form, such as substantially isolated form. By "isolated" polypeptide or protein is intended a polypeptide or protein removed from its native environment. For example, recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for the

purpose of the invention as are recombinant polypeptides which have been substantially purified by any suitable technique. A polypeptide variant according to the invention can be recovered and purified from recombinant cell cultures by methods known in the art.

Polypeptides of the present invention include products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or
5 eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial
10 modified methionine residue, in some cases as a result of host-mediated processes.

A polypeptide according to the present invention may be a fusion protein. Techniques for producing fusion polypeptides are known in the art, and include ligating the coding sequences encoding the polypeptides so that they are in frame. Expression of the fused polypeptide is under control of the same promoter (s) and terminator. The hybrid
15 polypeptides may comprise a combination of partial or complete polypeptide sequences obtained from at least two different polypeptides wherein one or more may be heterologous to a host cell. Such fusion polypeptides from at least two different polypeptides may comprise a binding domain from one polypeptide, such as a starch binding domain or a carbohydrate binding domain, operably linked to a catalytic domain from a second
20 polypeptide. Examples of fusion polypeptides and signal sequence fusions are for example as described in WO2010/121933, WO2013/007820 and WO2013/007821.

A polypeptide according to the present invention may comprise a catalytic domain and a binding domain, such as a starch or carbohydrate binding domains.

The invention also features biologically active fragments of the polypeptide
25 variants according to the invention. Such fragments are considered to be encompassed within the term "a variant of the invention".

Biologically active fragments of a polypeptide variant of the invention include polypeptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of a variant protein of the invention which include fewer amino
30 acids than the full length protein but which exhibit at least one biological activity of the corresponding full-length protein. Typically, biologically active fragments comprise a domain or motif with at least one activity of a variant protein of the invention. A biologically active fragment of a protein of the invention can be a polypeptide which is, for example, 10, 25, 50, 100 or more amino acids in length. Moreover, other biologically

active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the biological activities of the native form of a polypeptide of the invention.

Typically, a protein fragment of the invention will comprise one or more of the
5 substitutions defined herein.

The invention also features nucleic acid fragments which encode the above biologically active fragments (which biologically active fragments are themselves variants of the invention).

As set out above, the present invention provides polynucleotides encoding the
10 variant polypeptides of the invention. The invention also relates to an isolated polynucleotide encoding at least one functional domain of a polypeptide variant of the invention. Typically, such a domain will comprise one or more of the substitutions described herein.

In one embodiment of the invention, the nucleic acid sequence according to the
15 invention encodes a polypeptide, wherein the polypeptide is a variant comprising an amino acid sequence that has one or more truncation(s), and/or at least one substitution, deletion and/or insertion of an amino acid as compared to the parent alpha-amylase. Such a polypeptide will, however, typically comprise one or more of the substitutions described herein.

As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid
20 molecules which include an open reading frame encoding a variant as described herein. A gene may include coding sequences, non-coding sequences, introns and regulatory sequences. That is to say, a "gene", as used herein, may refer to an isolated nucleic acid molecule as defined herein. Accordingly, the term "gene", in the context of the present
25 application, does not refer only to naturally-occurring sequences.

A nucleic acid molecule of the present invention can be generated using standard molecular biology techniques well known to those skilled in the art taken in combination with the sequence information provided herein.

A nucleic acid molecule of the present invention can be adapted according to the method
30 described in patent application US090286280.

For example, using standard synthetic techniques, the required nucleic acid molecule may be synthesized *de novo*. Such a synthetic process will typically be an automated process.

Alternatively, a nucleic acid molecule of the invention may be generated by use of site-directed mutagenesis of an existing nucleic acid molecule, for example a wild-type nucleic acid molecule. Site-directed mutagenesis may be carried out using a number of techniques well known to those skilled in the art.

5 In one such method, mentioned here merely by way of example, PCR is carried out on a plasmid template using oligonucleotide "primers" encoding the desired substitution. As the primers are the ends of newly-synthesized strands, should there be a mis-match during the first cycle in binding the template DNA strand, after that first round, the primer-based strand (containing the mutation) would be at equal concentration to the
10 original template. After successive cycles, it would exponentially grow, and after 25, would outnumber the original, unmutated strand in the region of 8 million: 1, resulting in a nearly homogeneous solution of mutated amplified fragments. The template DNA may then be eliminated by enzymatic digestion with, for example using a restriction enzyme which cleaves only methylated DNA, such as Dpn1. The template, which is derived from
15 an alkaline lysis plasmid preparation and therefore is methylated, is destroyed in this step, but the mutated plasmid is preserved because it was generated *in vitro* and is unmethylated as a result.

 In such a method more than one mutation (encoding a substitution as described herein) may be introduced into a nucleic acid molecule in a single PCR
20 reaction, for example by using one or more oligonucleotides, each comprising one or more mis-matches. Alternatively, more than one mutation may be introduced into a nucleic acid molecule by carrying out more than one PCR reaction, each reaction introducing one or more mutations, so that altered nucleic acids are introduced into the nucleic acid in a sequential, iterative fashion.

25 A nucleic acid of the invention can be generated using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate mis-matched oligonucleotide primers according to the site-directed mutagenesis technique described above. A nucleic acid molecule derived in this way can be cloned into an appropriate vector and characterized by DNA sequence analysis.

30 A nucleic acid sequence of the invention may comprise one or more deletions, i.e. gaps, in comparison to the parent alpha-amylase. Such deletions/gaps may also be generated using site-directed mutagenesis using appropriate oligonucleotides. Techniques for generating such deletions are well known to those skilled in the art.

Furthermore, oligonucleotides corresponding to or hybridizable to nucleotide sequences according to the invention can be prepared by standard synthetic techniques, e.g. using an automated DNA synthesizer.

Also, complementary nucleic acid molecules are included in the present invention. A nucleic acid molecule which is complementary to another nucleotide sequence is one which is sufficiently complementary to the other nucleotide sequence such that it can hybridize to the other nucleotide sequence thereby forming a stable duplex.

One aspect of the invention pertains to isolated nucleic acid molecules that encode a variant of the invention, or a biologically active fragment or domain thereof, as well as nucleic acid molecules sufficient for use as hybridization probes to identify nucleic acid molecules encoding a polypeptide of the invention and fragments of such nucleic acid molecules suitable for use as PCR primers for the amplification or mutation of nucleic acid molecules, such as for the preparation of nucleic acid molecules of the invention.

An "isolated polynucleotide" or "isolated nucleic acid" is a DNA or RNA that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. Thus, in one embodiment, an isolated nucleic acid includes some or all of the 5' non-coding (e.g., promotor) sequences that are immediately contiguous to the coding sequence. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene encoding an additional polypeptide that is substantially free of cellular material, viral material, or culture medium (when produced by recombinant DNA techniques), or chemical precursors or other chemicals (when chemically synthesized). Moreover, an "isolated nucleic acid fragment" is a nucleic acid fragment that is not naturally occurring as a fragment and would not be found in the natural state.

As used herein, the terms "polynucleotide" or "nucleic acid molecule" are intended to include DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The

nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA. The nucleic acid may be synthesized using oligonucleotide analogs or derivatives (e.g., inosine or phosphorothioate nucleotides). Such oligonucleotides can be used, for example, to prepare nucleic acids that have altered
5 base-pairing abilities or increased resistance to nucleases.

Another embodiment of the invention provides an isolated nucleic acid molecule which is antisense to a nucleic acid molecule of the invention.

The terms "homology" or "percent identity" are used interchangeably herein. For the purpose of this invention, it is defined here that in order to determine the percent
10 identity of two amino acid sequences or two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid for optimal alignment with a second amino or nucleic acid sequence). The amino acid or nucleotide residues at corresponding amino acid or nucleotide positions are then compared. When a position in the first sequence is
15 occupied by the same amino acid or nucleotide residue as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = number of identical positions/total number of positions (i.e. overlapping positions) x 100). Preferably, the two sequences are the same
20 length.

A sequence comparison may be carried out over the entire lengths of the two sequences being compared or over fragment of the two sequences. Typically, the comparison will be carried out over the full length of the two sequences being compared. However, sequence identity may be carried out over a region of, for example, twenty,
25 fifty, one hundred or more contiguous amino acid residues.

The skilled person will be aware of the fact that several different computer programs are available to determine the homology between two sequences. For instance, a comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm.

30 The percent sequence identity between two amino acid sequences or between two nucleotide sequences may be determined using the Needleman and Wunsch algorithm for the alignment of two sequences. (Needleman, S. B. and Wunsch, C. D. (1970) J. Mol. Biol. 48, 443-453). Both amino acid sequences and nucleotide sequences can be aligned by the algorithm. The Needleman-Wunsch algorithm has been

implemented in the computer program NEEDLE. In an aspect the NEEDLE program from the EMBOSS package is used (version 2.8.0 or higher, EMBOSS: The European Molecular Biology Open Software Suite (2000) Rice,P. Longden,I. and Bleasby,A. Trends in Genetics 16, (6) p. 276-277, <http://emboss.bioinformatics.nl/>). In an aspect for protein sequences EBLOSUM62 is used for the substitution matrix. In an aspect for nucleotide sequences, EDNAFULL is used. The optional parameters used are a gap-open penalty of 10 and a gap extension penalty of 0.5. The skilled person will appreciate that all these different parameters will yield slightly different results but that the overall percentage identity of two sequences is not significantly altered when using different algorithms.

After alignment by the program NEEDLE as described above the percentage of sequence identity between a query sequence and a sequence of the invention is calculated as follows: Number of corresponding positions in the alignment showing an identical amino acid or identical nucleotide in both sequences divided by the total length of the alignment after subtraction of the total number of gaps in the alignment. The identity as defined herein can be obtained from NEEDLE by using the NOBRIEF option and is labeled in the output of the program as "longest-identity".

The protein sequences or nucleic acid sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403—10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, word length = 12 to obtain nucleotide sequences similar to nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, word length = 3 to obtain amino acid sequences similar to protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17): 3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See the homepage of the National Center for Biotechnology Information at <http://www.ncbi.nlm.nih.gov/>.

Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid encoding a variant alpha-amylase polypeptide of the invention.

As used herein, the term “vector” refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a “plasmid”, which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as “expression vectors”. In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. The terms “plasmid” and “vector” can be used interchangeably herein as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vector includes one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, “operatively linked” is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (e.g., in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell). The term “regulatory sequence” is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signal). Such regulatory sequences are described, for example, in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cells and those which direct expression of the nucleotide sequence only in a certain host cell (e.g. tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such

factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, encoded by nucleic acids as described herein (e.g. an alpha-amylase variant of SEQ ID NO: 2, for example a functional equivalent or
5 fragment, or a fusion protein comprising one or more of such variants).

The recombinant expression vectors of the invention can be designed for expression of variant proteins of the invention in prokaryotic or eukaryotic cells. For example, a variant protein of the invention can be expressed in bacterial cells such as E. coli, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells.
10 Suitable host cells are discussed further in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression vectors useful in the present invention include chromosomal-,
15 episomal- and virus-derived vectors e.g., vectors derived from bacterial plasmids, bacteriophage, yeast episome, yeast chromosomal elements, viruses such as baculoviruses, papova viruses, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as
20 cosmids and phagemids.

The DNA insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the E. coli lac, trp and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled person. In a specific embodiment, promoters are
25 preferred that are capable of directing a high expression level of alpha-amylase in filamentous fungi. Such promoters are known in the art. The expression constructs may contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and
30 a termination codon appropriately positioned at the end of the polypeptide to be translated.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized

techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, transduction, infection, lipofection, cationic lipid-mediated transfection or electroporation. Suitable methods for transforming or transfecting host cells can be found
5 in Sambrook, et al. (Molecular Cloning: A Laboratory Manual, 2nd, ed. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), Davis et al., Basic Methods in Molecular Biology (1986) and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may
10 integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be
15 introduced into a host cell on the same vector as that encoding a variant protein of the invention or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (e.g. cells that have incorporated the selectable marker gene will survive, while the other cells die).

Expression of proteins in prokaryotes is often carried out in *E. coli* with vectors
20 containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins.

The nucleic acid sequences according to the invention can be cloned in a suitable vector and after introduction in a suitable host, the sequence can be expressed to produce the corresponding alpha-amylase variants according to standard cloning and expression
25 techniques, which are known to the person skilled in the art (e. g., as described in (Sambrook & Russell, Molecular Cloning: A Laboratory Manual, 3rd Ed., CSHL Press, Cold Spring Harbor, NY, 2001). The invention also relates to such vectors comprising a nucleic acid sequence according to the invention.

Suitable vectors are the vectors normally used for cloning and expression and are known
30 to the person skilled in the art. Examples of suitable vectors for expression in *E. coli* are given e.g. in table 1 in Makrides, S. C., Microbiological Reviews, Vol. 60, No. 3, (1996), 512–538. Preferably, the vector contains a promoter upstream of the cloning site containing the nucleic acid sequence encoding the polypeptide with alpha-amylase activity, which can be switched on after the host has been grown to express the

corresponding polypeptide having alpha-amylase activity. Promoters, which can be switched on and off are known to the person skilled in the art and are for example the lac promoter, the aroH promoter, the araBAD promoter, the T7 promoter, the trc promoter, the tac promoter and the trp promoter. Particularly useful in the framework of the invention are for example the vectors as described in WO 00/66751, e.g. pKAFssECtrp or pKAFssECaro without the insert, the penicillin G acylase gene. Suitable hosts are the hosts normally used for cloning and expression and are known to the person skilled in the art. Examples of suitable host strains are for example *Escherichia coli* strains, e.g. *E. coli* TOP10F', TOP10, DH10B, DH5a, HB101, W3110, BL21(DE3) and BL21 (DE3)pLysS. Particularly useful in the framework of the invention are *Escherichia coli* K-12 strains, e.g. DH1, HB101, RV308, RR1, W3110, C600 and/or derivatives of these strains. The choice of the vector can sometimes depend on the choice of the host and vice versa. If e.g. a vector with the araBAD promoter is being used, an *E. coli* host strain that is unable to break down the arabinose inducer (ara-), is strongly preferred.

There are several ways of inserting a nucleic acid into a nucleic acid construct or an expression vector which are known to a skilled person in the art. It may be desirable to manipulate a nucleic acid encoding a polypeptide of the present invention with control sequences, such as promoter and terminator sequences.

A promoter may be any appropriate promoter sequence suitable for a eukaryotic or prokaryotic host cell, which shows transcriptional activity, including mutant, truncated, and hybrid promoters, and may be obtained from polynucleotides encoding extracellular or intracellular polypeptides either endogenous (native) or heterologous (foreign) to the cell. The promoter may be a constitutive or inducible promoter. Preferably, the promoter is an inducible promoter, for instance a starch inducible promoter. Promoters suitable in filamentous fungi are promoters which may be selected from the group, which includes but is not limited to promoters obtained from the polynucleotides encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus gpdA* promoter, *A. niger* neutral alpha-amylase, *A. niger* acid stable alpha-amylase, *A. niger* or *A. awamori* glucoamylase (glaA), *A. niger* or *A. awamori* endoxylanase (xlnA) or beta-xylosidase (xlnD), *T. reesei* cellobiohydrolase I (CBHI), *R. miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase, *A. nidulans* acetamidase, *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Dania (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei*

cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase IV, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* beta-xylosidase, as well as the NA2-tpi promoter (a hybrid of the promoters from the polynucleotides encoding *A. niger* neutral alpha-amylase and *A. oryzae* triose phosphate isomerase), and mutant, truncated, and hybrid promoters thereof. Other examples of promoters are the promoters described in WO2006/092396 and WO2005/100573, which are herein incorporated by reference. Suitable promoters in eukaryotic host cells may be *GAL7*, *GAL10*, or *GAL1*, *CYC1*, *HIS3*, *ADH1*, *PGL*, *PH05*, *GAPDH*, *ADC1*, *TRP1*, *URA3*, *LEU2*, *ENO1*, *TPI1*, and *AOX1*. Other suitable promoters include *PDC1*, *GPD1*, *PGK1*, *TEF1*, and *TDH3*. All of the above-mentioned promoters are readily available in the art.

Suitable inducible promoters useful in bacteria, such as Bacilli, include: promoters from Gram-positive microorganisms such as, but are not limited to, SP01-26, SP01-15, veg, pyc (pyruvate carboxylase promoter), and amyE. Examples of promoters from Gram-negative microorganisms include, but are not limited to, tac, tet, trp-tet, lpp, lac, lpp-lac, lacIq, P15, T7, T5, T3, gal, trc, ara, SP6, λ -PR, and λ -PL.

Any terminator which is functional in a cell as disclosed herein may be used, which are known to a skilled person in the art. Examples of suitable terminator sequences in filamentous fungi include terminator sequences of a filamentous fungal gene, such as from *Aspergillus* genes, for instance from the gene *A. oryzae* TAKA amylase, the genes encoding *A. niger* glucoamylase (glaA), *A. nidulans* anthranilate synthase, *A. niger* alpha-glucosidase, trpC and/or *Fusarium oxysporum* trypsin-like protease

Fusion vectors add a number of amino acids to a protein encoded therein, e.g. to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein.

As indicated, the expression vectors will preferably contain selectable markers. Such markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline or ampicillin resistance for culturing in *E. coli* and other bacteria. Representative examples of appropriate host include bacterial cells, such as *E. coli*,
5 *Streptomyces* *Salmonella typhimurium* and certain *Bacillus* species; fungal cells such as *Aspergillus* species, for example *A. niger*, *A. oryzae* and *A. nidulans*, such as yeast such as *Kluyveromyces*, for example *K. lactis* and/or *Pichia*, for example *P. pastoris*; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9; animal cells such as CHO, COS and Bowes melanoma; and plant cells. Appropriate culture mediums and conditions for the
10 above-described host cells are known in the art.

Vectors preferred for use in bacteria are for example disclosed in WO-A1-2004/074468, which are hereby enclosed by reference. Other suitable vectors will be readily apparent to the skilled artisan.

Known bacterial promoters suitable for use in the present invention include the
15 promoters disclosed in WO-A1-2004/074468, which are hereby incorporated by reference.

Transcription of the DNA encoding a variant of the present invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act to
20 increase transcriptional activity of a promoter in a given host cell-type. Examples of enhancers include the SV40 enhancer, which is located on the late side of the replication origin at bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

For secretion of the translated protein into the lumen of the endoplasmic
25 reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signal may be incorporated into the expressed polypeptide. The signals may be endogenous to the polypeptide or they may be heterologous signals.

A variant of the invention may be expressed in form such that it may include additional heterologous functional regions, for example secretion signals. A variant of the
30 invention may also comprise, for example, a region of additional amino acids, particularly charged amino acids, added to the N-terminus of the polypeptide for instance to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, peptide moieties may be added to a variant of the invention to facilitate purification, for example by the addition of histidine residues or a T7 tag.

The variants of the invention, such as proteins of the present invention or functional equivalents thereof, e.g., biologically active portions and fragments thereof, can be operatively linked to a non-variant polypeptide (e.g., heterologous amino acid sequences) to form fusion proteins. A "non-variant polypeptide" in this context refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to a variant alpha-amylase of the invention.

Within a fusion protein, the variant of the invention can correspond to a full length sequence or a biologically active fragment of a polypeptide of the invention. In a preferred embodiment, a fusion protein of the invention comprises at least two biologically active portions. Within the fusion protein, the term "operatively linked" is intended to indicate that the variant polypeptide and the non-variant polypeptide are fused in-frame to each other. The non-variant polypeptide can be fused to the N-terminus or C-terminus of the variant polypeptide.

Expression and secretion of a variant alpha-amylase may be enhanced by expressing the variant in the form of a fusion protein. In this context, a nucleic acid sequence may encode for a fusion protein comprising pre-alpha-amylase or alpha-amylase. More specifically, the fusion partner may be glucoamylase or a fragment thereof. In one embodiment the pre-alpha-amylase or alpha-amylase, or a fusion protein thereof, is secreted over the host cell membrane.

For example, in one embodiment, the fusion protein is a fusion protein in which the variant sequence/s is/are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of a recombinant variant according to the invention. In another embodiment, the fusion protein is a variant of the invention containing a heterologous signal sequence at its N-terminus. In certain host cells (e.g., mammalian and yeast host cells), expression and/or secretion of a variant of the invention can be increased through use of a heterologous signal sequence.

In another example, the gp67 secretory sequence of the baculovirus envelope protein can be used as a heterologous signal sequence (Current Protocols in Molecular Biology, Ausubel et al., eds., John Wiley & Sons, 1992). Other examples of eukaryotic heterologous signal sequences include the secretory sequences of melittin and human placental alkaline phosphatase (Stratagene; La Jolla, California). In yet another example, useful prokaryotic heterologous signal sequences include the phoA secretory signal (Sambrook et al., supra) and the protein A secretory signal (Pharmacia Biotech; Piscataway, New Jersey).

A signal sequence can be used to facilitate secretion and isolation of a variant of the invention. Signal sequences are typically characterized by a core of hydrophobic amino acids, which are generally cleaved from the mature protein during secretion in one or more cleavage events. Such signal peptides contain processing sites that allow
5 cleavage of the signal sequence from the mature proteins as they pass through the secretory pathway. The signal sequence may direct secretion of the variant, such as from a eukaryotic host into which the expression vector is transformed, and the signal sequence may then be subsequently or concurrently cleaved. The variant of the invention may then be readily purified from the extracellular medium by known methods.
10 Alternatively, the signal sequence can be linked to the variant of interest using a sequence, which facilitates purification, such as with a GST domain. Thus, for instance, the sequence encoding the variant of the invention may be fused to a marker sequence, such as a sequence encoding a peptide, which facilitates purification of the fused variant of the invention. In certain preferred embodiments of this aspect of the invention, the
15 marker sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz et al, Proc. Natl. Acad. Sci. USA 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The HA tag is another peptide useful for purification which corresponds to an epitope derived of influenza hemagglutinin
20 protein, which has been described by Wilson et al., Cell 37:767 (1984), for instance.

A fusion protein of the invention may be produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction
25 enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers, which give rise to
30 complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, Current Protocols in Molecular Biology, eds. Ausubel et al. John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g, a GST polypeptide). A variant-encoding nucleic

acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the said variant.

The terms “functional equivalents” and “functional variants” are used interchangeably herein. Functional equivalents according to the invention are isolated
5 DNA fragments that encode a polypeptide that exhibits a particular function of a variant as defined herein. Functional equivalents therefore also encompass biologically active fragments and are themselves encompassed within the term “a variant” of the invention.

Preferably, a functional equivalent of the invention comprises one or more of the substitutions described herein. However, a functional equivalent may comprise one or
10 more modifications in addition to the substitutions described above.

Functional nucleic acid equivalents may typically contain silent mutations or mutations that do not alter the biological function of encoded polypeptide. Accordingly, the invention provides nucleic acid molecules encoding a variant alpha-amylase protein that contains changes in amino acid residues that are not essential for a particular
15 biological activity. Such variant proteins differ in amino acid sequence from the parent alpha-amylase sequence from which they are derived yet retain at least one biological activity thereof, preferably they retain at least alpha-amylase activity. In one embodiment the isolated nucleic acid molecule comprises a nucleotide sequence encoding a protein, wherein the protein comprises a substantially homologous amino acid sequence of at
20 least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the reference amino acid sequence (for example that shown in SEQ ID NO: 2).

As defined herein, the term “substantially homologous” refers to a first amino acid or nucleotide sequence which contains a sufficient or minimum number of identical
25 or equivalent (e.g., with similar side chain) amino acids or nucleotides to a second amino acid or nucleotide sequence such that the first and the second amino acid or nucleotide sequences have a common domain. For example, amino acid or nucleotide sequences which contain a common domain having about 60%, preferably 65%, more preferably 70%, even more preferably 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity
30 or more are defined herein as sufficiently identical.

The skilled person will recognise that changes can be introduced by mutation into the nucleotide sequences according to the invention thereby leading to changes in the amino acid sequence of the resulting protein without substantially altering the function of such a protein.

Accordingly, an alpha-amylase variant of the invention is preferably a protein which comprises an amino acid sequence at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the reference amino acid sequence, for example that shown in SEQ ID NO: 2, and typically also retains at least one functional activity of the reference polypeptide. Variants of the invention, for example functional equivalents of a protein according to the invention, can also be identified e.g. by screening combinatorial libraries of mutants, e.g. truncation mutants, of the protein of the invention for alpha-amylase activity. In one embodiment, a variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level. A variegated library of variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential protein sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display). There are a variety of methods that can be used to produce libraries of potential variants of the polypeptides of the invention from a degenerate oligonucleotide sequence. Methods for synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang (1983) Tetrahedron 39:3; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477).

According to one embodiment of the invention, the recombinant host cell is preferably capable of expressing or overexpressing the nucleotide sequence or nucleic acid molecule according to the invention, the mutant microbial host cell may further comprise one or more modifications in its genome such that the mutant microbial host cell is deficient in the production of at least one product selected from glucoamylase (*glaA*), acid stable alpha-amylase (*amyA*), neutral alpha-amylase (*amyBI* and *amyBII*), oxalic acid hydrolase (*oahA*), a toxin, preferably ochratoxin and/or fumonisin, a protease transcriptional regulator *prtT*, *PepA*, a product encoded by the gene *hdfA* and/or *hdfB*, a non-ribosomal peptide synthase *npsE*, *agsE* or *amyC* if compared to a parent host cell and measured under the same conditions. Suitable methods of producing said host cells include the ones described in WO2011/009700, WO2012/001169, WO2014013074.

In addition, libraries of fragments of the sequence encoding a polypeptide of the invention can be used to generate a variegated population of polypeptides for screening a subsequent selection of variants. For example, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of the coding sequence of interest with a nuclease under conditions wherein nicking occurs only about once per

molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal and internal fragments of various sizes of the protein of interest.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations of truncation, and for screening cDNA libraries for gene products having a selected property. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify variants of a protein of the invention (Arkin and Yourvan (1992) Proc. Natl. Acad. Sci. USA 89:7811-7815; Delgrave et al. (1993) Protein Engineering 6(3): 327-331).

Fragments of a polynucleotide according to the invention may also comprise polynucleotides not encoding functional polypeptides. Such polynucleotides may function as probes or primers for a PCR reaction.

Nucleic acids according to the invention irrespective of whether they encode functional or non-functional polypeptides can be used as hybridization probes or polymerase chain reaction (PCR) primers. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having alpha-amylase activity include, inter alia, (1) *in situ* hybridization (e.g. FISH) to metaphase chromosomal spreads to provide precise chromosomal location of an alpha-amylase-encoding gene as described in Verma *et al.*, Human Chromosomes: a Manual of Basic Techniques, Pergamon Press, New York (1988); (2) Northern blot analysis for detecting expression of alpha-amylase mRNA in specific tissues and/or cells; and (3) probes and primers that can be used as a diagnostic tool to analyse the presence of a nucleic acid hybridizable to such a probe or primer in a given biological (e.g. tissue) sample.

Variants of a given reference alpha-amylase enzyme can be obtained by the following standard procedure:

- Mutagenesis (error-prone, doped oligo, spiked oligo) or synthesis of variants
- Transformation in, for example *B. subtilis*
- Cultivation of transformants, selection of transformants
- Expression
- 5 - Optional purification and concentration
- Primary Screening
- Identification of an improved variant (for example in relation to specific activity)
- and characterization of the variation(s) and/or mutation(s) within the alpha-amylase enzyme identified.

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In another embodiment, the invention features cells, e.g., transformed host cells or recombinant host cells that contain a nucleic acid encompassed by the invention. A "transformed cell" or "recombinant cell" is a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a nucleic acid

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according to the invention. Both prokaryotic and eukaryotic cells are included, e.g., bacteria, fungi, yeast, and the like, especially preferred are cells from yeasts, for example, *K. lactis*. Host cells also include, but are not limited to, mammalian cell lines such as CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and choroid plexus cell lines.

20

Examples of suitable bacterial host organisms are gram positive bacterial species such as *Bacillaceae* including *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium* and *Bacillus thuringiensis*, *Streptomyces* species such as *Streptomyces murinus*, lactic acid bacterial species including *Lactococcus spp.* such as *Lactococcus lactis*, *Lactobacillus spp.* including *Lactobacillus reuteri*, *Leuconostoc spp.* and *Streptococcus spp.* Alternatively, strains of a gram negative bacterial species such as a species belonging to *Enterobacteriaceae*, including *E. coli* or to *Pseudomonadaceae* may be selected as the host organism.

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A suitable yeast host organism may advantageously be selected from a species of *Saccharomyces* including *Saccharomyces cerevisiae* or a species belonging to *Schizosaccharomyces*. Further useful yeast host organisms include *Pichia spp.* such as methylotrophic species hereof, including *Pichia pastoris*, and *Kluyveromyces spp.* including *Kluyveromyces lactis*.

Suitable host organisms among filamentous fungi include species of *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Rasamsonia*, *Thielavia*, *Tolypocladium* or *Trichoderma*, such as e. g. *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus oryzae*, *Aspergillus nidulans* or *Aspergillus niger*, including *Aspergillus nigervar. awamori*, *Fusarium bactridioides*, *Fusarium cereals*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola langinosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium chrysogenum*, *Penicillium camembertii*, *Penicillium purpurogenum*, *Rasamsonia emersonii*, *Rhizomucor miehei*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesii* or *Trichoderma viride*.

A host cell can be chosen that modulates the expression of the inserted sequences, or modifies and processes the product encoded by the incorporated nucleic acid sequence in a specific, desired fashion. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may facilitate optimal functioning of the encoded protein.

Various host cells have characteristic and specific mechanisms for post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems familiar to those of skill in the art of molecular biology and/or microbiology can be chosen to ensure the desired and correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product can be used. Such host cells are well known in the art.

If desired, a stably transfected cell line can produce a variant according to the invention. A number of vectors suitable for stable transfection of mammalian cells are available to the public, methods for constructing such cell lines are also publicly known, e.g., in Ausubel et al. (supra).

The present invention also provides a nucleic acid sequence encoding the variant polypeptides of the invention. The invention thus also provides a nucleic acid sequence encoding a variant polypeptide having alpha-amylase activity which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue corresponding to any of amino acids

14, 15, 124, 133, 225, 200, 282, 358,

said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention thus also provides a nucleic acid sequence encoding a variant polypeptide having alpha-amylase activity which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 and at least one further substitution of an amino acid residue corresponding to any of amino acids

14, 15, 124, 133, 200, 282, 358,

said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The invention thus also provides a nucleic acid sequence encoding a variant polypeptide having alpha-amylase activity which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 and at least one further substitution of an amino acid residue corresponding to any of amino acids

14, 15, 70, 124, 133, 225, 282, 358,

said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The present invention also provides a nucleic acid construct comprising the nucleic acid sequence of the invention operably linked to one or more control sequences capable of directing the expression of an alpha-amylase in a suitable expression host.

The present invention also provides a recombinant expression vector comprising the nucleic acid construct of the invention.

The present invention also provides a recombinant host cell comprising the expression vector of the invention.

5 The present invention also provides a method for producing an alpha-amylase comprising cultivating the host cell of the invention under conditions conducive to production of the alpha-amylase and recovering the alpha-amylase.

10 The present invention also provides a method of producing an alpha-amylase polypeptide variant according to the invention, which method comprises:

- a) selecting a reference alpha-amylase polypeptide;
- b) substituting at least one amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue corresponding to any of amino acids

15 14, 15, 124, 133, 225, 200, 282, 358,

said positions being defined with reference to SEQ ID NO: 2;

- c) optionally substituting one or more further amino acids as defined in b);
- d) preparing the variant resulting from steps a)-c);
- e) determining a property of the variant; and
- 20 f) selecting a variant having an altered property in comparison to the reference alpha-amylase polypeptide. In a preferred embodiment of the method of producing the alpha-amylase polypeptide variant according to the invention, the reference polypeptide has the sequence as set in SEQ ID NO: 2. The reference polypeptide may have a sequence having at least 70% identity to the sequence as set in SEQ ID NO: 2.

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The present invention also provides a composition comprising the variant polypeptide according to the invention or obtainable by a method according to the invention and one or more components selected from the group consisting of milk, gluten, granulated fat, an additional enzyme, an amino acid, a salt, oxidants, reducing agents, 30 emulsifiers, gums, flavours, acids, starch, modified starch, humectants and preservatives.

A composition according to the invention comprises the variant polypeptide of the invention.

The present invention further discloses a composition comprising the alpha-amylase variants according to the invention and one or more components selected from

the group consisting of milk (including liquid milk and milk powder), gluten, granulated fat, an additional enzyme, an amino acid, a salt, oxidants (including ascorbic acid, bromate and Azodicarbonamide (ADA), reducing agents (including L-cysteine), emulsifiers (including without limitation mono- and diglycerides, monoglycerides such as
5 glycerol monostearate (GMS), sodium stearyl lactylate (SSL), calcium stearyl lactylate (CSL), polyglycerol esters of fatty acids (PGE) and diacetyl tartaric acid esters of mono- and diglycerides (DATEM), propylene glycol monostearate (PGMS), lecithin), gums (including guar gum and xanthan gum), flavours, acids (including citric acid, propionic acid), starch, modified starch, humectants (including glycerol) and preservatives.

10 In an aspect of the composition according to the invention the additional enzyme may include including a further alpha-amylase, such as a fungal alpha-amylase (which may be useful for providing sugars fermentable by yeast and retarding staling), beta-amylase, a cyclodextrin glucanotransferase, a protease, a peptidase, in particular, an exopeptidase (which may be useful in flavour enhancement), transglutaminase, triacyl
15 glycerol lipase (which may be useful for the modification of lipids present in the dough or dough constituents so as to soften the dough), galactolipase, phospholipase, cellulase, hemicellulase, in particular a pentosanase such as xylanase (which may be useful for the partial hydrolysis of pentosans, more specifically arabinoxylan, which increases the extensibility of the dough), protease (which may be useful for gluten weakening in
20 particular when using hard wheat flour), protein disulfide isomerase, e.g., a protein disulfide isomerase as disclosed in WO 95/00636, glycosyltransferase, peroxidase (which may be useful for improving the dough consistency), laccase, or oxidase, hexose oxidase, e.g., a glucose oxidase, aldose oxidase, pyranose oxidase, lipoxygenase or L-amino acid oxidase (which may be useful in improving dough consistency), a protease or
25 an asparaginase.

In an embodiment of the composition according to the invention the additional enzyme is a lipolytic enzyme.

A lipolytic enzyme, also referred to herein as lipase, is an enzyme that hydrolyses triacylglycerol and/or galactolipid and/or phospholipids.

30 The specificity of the lipase can be shown through in vitro assay making use of appropriate substrate, for example triacylglycerol lipid, phosphatidylcholine and digalactosyldiglyceride, or preferably through analysis of the reactions products that are generated in the dough during mixing and fermentation.

The triacyl glycerol lipase may be a fungal lipase, preferably from *Rhizopus*, *Aspergillus*, *Candida*, *Penicillium*, *Thermomyces*, or *Rhizomucor*. In an embodiment the triacyl glycerol lipase is from *Rhizopus*, in a further embodiment a triacyl glycerol lipase from *Rhizopus oryzae* is used. Optionally a combination of two or more triacyl glycerol lipases may be used

In an embodiment of the composition according to the invention the additional enzyme is a phospholipase.

In an embodiment of the composition according to the invention the additional enzyme is a galactolipase.

In an embodiment of the enzyme composition according to the invention the additional enzyme is an enzyme having both phospholipase and galactolipase activity.

A phospholipase is an enzyme that catalyzes the release of fatty acyl groups from a phospholipid. It may be a phospholipase A2 (PLA2, EC 3.1.1.4) or a phospholipase A1 (EC 3.1.1.32). It may or may not have other activities such as triacylglycerol lipase (EC 3.1.1.3) and/or galactolipase (EC 3.1.1.26) activity.

The phospholipase may be a native enzyme from mammalian or microbial sources. An example of a mammalian phospholipase is pancreatic PLA2, e.g. bovine or porcine PLA2 such as the commercial product Lecitase 10L (porcine PLA2, product of Novozymes A/S).

Microbial phospholipases may be from *Fusarium*, e.g. *F. oxysporum* phospholipase A1 (WO 1998/026057), *F. venenatum* phospholipase A1 (described in WO 2004/097012 as a phospholipase A2 called FvPLA2), from *Tuber*, e.g. *T. borchii* phospholipase A2 (called TbPLA2, WO 2004/097012).

The phospholipase may also be a lipolytic enzyme variant with phospholipase activity, e.g. as described in WO 2000/032758 or WO 2003/060112 .

The phospholipase may also catalyze the release of fatty acyl groups from other lipids present in the dough, particularly wheat lipids. Thus, the phospholipase may have triacylglycerol lipase activity (EC 3.1.1.3) and/or galactolipase activity (EC 3.1.1.26).

The phospholipase may be a lipolytic enzyme as described in WO2009/106575, such as the commercial product Panamore®, product of DSM.

Panamore®, Lipopan® F, Lipopan® 50 and Lipopan® S are commercialised to standardised lipolytic activity, using a measurement of DLU for Panamore® from DSM and a measurement of LU for the Lipopan® family from Novozymes. DLU is defined as the amount of enzyme needed to produce 1 micromol/min of p-nitrophenol from p-

nitrophenyl palmitate at pH 8.5 at 37°C, while LU is defined as the amount of enzyme needed to produce 1micromol/min of butyric acid from tributyrin at pH 7 at 30 °C. Lipases are optimally used with the alpha-amylase of the invention at 2-850 DLU/kg flour or at 50-23500 LU/kg flour.

5 The cellulase may be from *A. niger* or from *Trichoderma reesei*.

 The amyloglucosidase, may be an amyloglucosidase from *Aspergillus* such as from *A. oryzae* or *A. niger*, preferably from *A. niger*.

 The additional enzyme may include without limitation an enzyme as disclosed in any of US 4,598,048; US 4,604,355; US RE38,507; WO99/43794; WO99/43794, in
10 particular in EP1058724B1; WO2004/081171; WO2006/012899; WO2008/148845; WO2006/032281 .

 The additional enzyme may include without limitation an enzyme as disclosed in any of US8426182; WO2013113665; non-prepublished European patent application having a filing number EP13183144.8.

15 The additional enzyme may include a G4-forming amylase.

A G4-forming amylase is an enzyme that is inter alia capable of catalysing the degradation of starch. In particular it is capable of cleaving α -D-(1 $\rightarrow$ 4) O-glycosidic linkages in starch. It may be referred to as a glucan 1,4-alpha-maltotetrahydrolase (EC 3.2.1.60). It may also be referred as a maltotetrahydrolase.

20 *Pseudomonas saccharophila* (GenBank Acc. No. X16732) expresses a G4-forming amylase. The G-4 forming amylase is capable of producing maltotetraose from either liquefied starch or other source of maltodextrins at a high temperature e.g. about 60 °C to about 75 °C. Suitable G4-forming amylases may be G4-forming amylases described in any one of WO9950399, WO2005007818, WO2004111217, WO2005003339,
25 WO2005007818, WO2005007867, WO2006003461, WO2007007053, WO2007148224, WO2009083592, WO2009088465.

 In an embodiment the enzyme composition according to the invention is provided in a dry form, to allow easy addition to the dough, the dough ingredients, but liquid forms are also possible. A liquid form includes without limitation an emulsion, a suspension and
30 a solution. Irrespective of the formulation of the enzyme composition, any additive or additives known to be useful in the art to improve and/or maintain the enzyme's activity, the quality of a dough and/or a baked product may be applied.

The present invention also provides a pre-mix comprising flour and the variant polypeptide according to the invention or obtainable by the method of the invention.

The alpha-amylase variant according to the invention may be incorporated in a pre-mix, e.g. in the form of a flour composition, for dough and/or baked products made from dough, in which the pre-mix comprises a polypeptide of the present invention.

The term "pre-mix" is defined herein to be understood in its conventional meaning, i.e. as a mix of baking agents, generally including flour, which may be used not only in industrial bread-baking plants/facilities, but also in retail bakeries. The pre-mix may be prepared by mixing the alpha-amylase polypeptide variant according to the invention or the enzyme composition according to the invention with a suitable carrier such as flour, starch or a salt. The pre-mix may contain additives as mentioned herein.

Additives are in most cases added in powder form. Suitable additives include oxidants (including ascorbic acid, bromate and Azodicarbonamide (ADA), reducing agents (including L-cysteine), emulsifiers (including without limitation mono- and diglycerides, monoglycerides such as glycerol monostearate (GMS), sodium stearyl lactylate (SSL), calcium stearyl lactylate (CSL), polyglycerol esters of fatty acids (PGE) and diacetyl tartaric acid esters of mono- and diglycerides (DATEM), propylene glycol monostearate (PGMS), lecithin), gums (including guar gum and xanthan gum), flavours, acids (including citric acid, propionic acid), starch, modified starch, humectants (including glycerol) and preservatives.

For inclusion in a pre-mix of flour it is advantageous that the (isolated) polypeptide according to the invention is in the form of a dry product, e.g., a non-dusting granulate, whereas for inclusion together with a liquid it is advantageously in a liquid form.

The invention also relates to the use of the alpha-amylase variant according to the invention in a number of industrial processes. Despite the long-term experience obtained with these processes, the alpha-amylase according to the invention may feature advantages over the enzymes currently used. Depending on the specific application, these advantages may include aspects like lower production costs, higher specificity towards the substrate, less antigenic, less undesirable side activities, higher yields when produced in a suitable microorganism, more suitable pH and temperature ranges, better tastes of the final product as well as food grade and kosher aspects.

In an embodiment the alpha-amylase variant according to the invention is used in the food industry, including in food manufacturing.

The invention provides the use of the variant polypeptide according to the invention or of the composition according to the invention or of the pre-mix according to the invention in the food industry.

An example of an industrial application of the alpha-amylase variant according to the invention in food is its use in baking applications. The alpha-amylase according to the invention may for example be used in baked products such as bread or cake. For example to improve quality of the dough and/or the baked product.

The present invention also provides a use of a variant polypeptide according to the invention or of the composition according to the invention or of the pre-mix according to the invention in the preparation of a dough and/or a baked product.

In an aspect, the alpha-amylase variant according to the invention, the composition according to the invention or the pre-mix according to the invention may be used in the production of cake and in the production of a batter from which a cake can be made.

The variant polypeptide according to the invention, enzyme composition according to the invention or the pre-mix according to the invention may be used for retarding staling of a baked product such as bread and/or cake. Retarding of staling may be indicated by a reduced hardness, in particular a reduced hardness after storage of a baked product prepared with the alpha-amylase variant compared to a baked product that is prepared with a parent polypeptide.

The present invention also provides a dough comprising the variant polypeptide according to the invention or of the composition according to the invention or of the pre-mix according to the invention.

A method of preparing a dough according to the invention comprises adding the alpha-amylase variant polypeptide according to the invention to the dough wherein the dough comprises at least 2 wt% sugar based on total recipe weight. In a further aspect of this method the dough comprises at least 3 wt% sugar, in an aspect comprises at least 5 wt% sugar, in an aspect at least 8 wt% sugar, in an aspect at least 12 wt% sugar, in an aspect at least 15 wt% sugar based on total recipe weight. In a further aspect of this embodiment the baked product comprises at least 18 wt% sugar, in an aspect at

least 20 wt% sugar, in an aspect at least 25 wt% sugar, in an aspect at least 30 wt% sugar based on total recipe weight.

The term "dough" is defined herein as a mixture of flour and other ingredients. In one aspect the dough is firm enough to knead or roll. The dough may be fresh, frozen
5 or prepared. The preparation of frozen dough is described by Kulp and Lorenz in Frozen and Refrigerated Doughs and Batters.

Dough is usually made using basic dough ingredients including (cereal) flour, such as wheat flour or rice flour, water and optionally salt. For leavened products, primarily baker's yeast is used next to chemical leavening systems such as a combination of an acid
10 (generating compound) and bicarbonate.

Dough ingredients, which include without limitation (cereal) flour, egg, water, salt, sugar, flavours, fat (including butter, margarine, oil and shortening), baker's yeast, a chemical leavening system such as a combination of an acid (generating compound) and bicarbonate, milk (including liquid milk and milk powder), soy flour, oxidants (including
15 ascorbic acid, bromate and Azodicarbonamide (ADA), reducing agents (including L-cysteine), emulsifiers (including mono/di glycerides, mono glycerides such as glycerol monostearate (GMS), sodium stearyl lactylate (SSL), calcium stearyl lactylate (CSL), polyglycerol esters of fatty acids (PGE) and diacetyl tartaric acid esters of mono- and diglycerides (DATEM) propylene glycol monostearate (PGMS), lecithin), gums (including
20 guar gum and xanthan gum), flavours, acids (including citric acid, propionic acid), starch, modified starch, humectants (including glycerol) and preservatives

Cereals include maize, rice, wheat, barley, sorghum, millet, oats, rye, triticale, buckwheat, quinoa, spelt, einkorn, emmer, durum and kamut.

The preparation of a dough from the dough ingredients is well known in the art and includes
25 mixing of said ingredients and optionally one or more moulding and fermentation steps.

Preparing a dough according to the invention may comprise the step of combining the alpha-amylase variant according to the invention or the composition according to the invention or the pre-mix according to the invention and at least one dough ingredient.

Combining' includes without limitation, adding the alpha-amylase variant according to the
30 invention or the composition according to the invention to the at least one component indicated above, adding the at least one component indicated above to the alpha-amylase variant according to the invention or the composition according to the invention, mixing the alpha-amylase variant according to the invention or the composition according to the invention and the at least one component indicated above.

'Combining' includes without limitation, adding the pre-mix according to the invention to the at least one dough ingredient, adding the at least one dough ingredient to the pre-mix according to the invention, mixing the pre-mix according to the invention and the at least one dough ingredient.

5 Yeast, enzymes and optionally additives are generally added separately to the dough. Enzymes may be added in a dry, e.g. granulated form, in a liquid form or in the form of a paste.

10 The term dough herein includes a batter. A batter is a semi-liquid mixture, being thin enough to drop or pour from a spoon, of one or more flours combined with liquids such as water, milk or eggs used to prepare various foods, including cake. The dough may be made using a mix including a cake mix, a biscuit mix, a brownie mix, a bread mix, a pancake mix and a crepe mix.

15 The term dough includes frozen dough, which may also be referred to as refrigerated dough. There are different types of frozen dough; that which is frozen before proofing and that which is frozen after a partial or complete proofing stage. The frozen dough is typically used for manufacturing baked products including without limitation biscuits, breads, bread sticks and croissants.

20 The present invention also relates to methods for preparing a dough or a baked product comprising incorporating into the dough an effective amount of the alpha-amylase variant, which improves one or more properties of the dough or the baked product obtained from the dough relative to a dough or a baked product in which a parent polypeptide is incorporated.

25 The phrase "incorporating into the dough" is defined herein as adding the alpha-amylase variant or a parent polypeptide to the dough, any ingredient from which the dough is to be made, and/or any mixture of dough ingredients from which the dough is to be made. In other words, the alpha-amylase variant or a parent polypeptide may be added in any step of the dough preparation and may be added in one, two or more steps. The alpha-amylase variant or a parent polypeptide to the dough are added to the ingredients of a
30 dough that is kneaded and baked to make the baked product using methods well known in the art. See, for example, U.S. Patent No. 4,567,046, EP-A-426,211, JP-A-60-78529, JP-A-62-111629, and JP-A-63-258528.

 The term "effective amount" is defined herein as an amount of the alpha-amylase variant that is sufficient for providing a measurable effect on at least one

property of interest of the dough and/or baked product. A suitable amount of alpha-amylase variant is in a range of 0.5-1500 NBAU/kg flour, in an embodiment 5-200 NBAU/kg flour, in a further embodiment 20-100 NBAU/kg flour. A suitable amount includes 1 ppm – 2000 ppm of an enzyme product having an activity in a range of about 700 to 1100 NBAU/g. In an embodiment an effective amount is in a range of 10 -200 ppm of an enzyme product having an activity in a range of about 700 to 1100 NBAU/g, in another embodiment 30-100 ppm of an enzyme product having an activity in a range of about 700 to 1100 NBAU/g. In an embodiment an effective amount is in a range of 10 - 200 ppm of an enzyme product having an activity of about 700 to 1100 NBAU/g. Herein and hereinafter NBAU stands for New Baking Amylase Unit as defined in the examples under the heading NBAU Assay

If one or more additional enzyme activities are to be added in accordance with the methods of the present invention, these activities may be added separately or together with the polypeptide according to the invention, for example as the enzyme composition according to the invention, which includes a bread-improving composition and/or a dough-improving composition. The other enzyme activities may be any of the enzymes described above and may be dosed in accordance with established baking practices.

The present invention also provides a process for the production of a baked product, which method comprises baking the dough according to the invention.

The term 'baked product' refers to a baked food product prepared from a dough. Examples of baked products, whether of a white, brown or whole-meal type, which may be advantageously produced by the present invention include bread (in particular white, whole-meal or rye bread), typically in the form of loaves or rolls, French baguette-type bread, pastries, croissants, brioche, panettone, pasta, noodles (boiled or (stir-)fried), pita bread and other flat breads, tortillas, tacos, cakes, pancakes, cookies in particular biscuits, doughnuts, including yeasted doughnuts, bagels, pie crusts, steamed bread, crisp bread, brownies, sheet cakes, snack foods (e.g., pretzels, tortilla chips, fabricated snacks, fabricated potato crisps). The term baked product includes without limitation, bread containing from 2 to 30 wt% sugar based on total recipe weight, fruit containing bread, breakfast cereals, cereal bars, eggless cake, soft rolls and gluten-free bread.

The term baked product includes without limitation, bread containing from 2 to 30 wt% sugar based on total recipe weight, fruit containing bread, breakfast cereals, cereal bars, eggless cake, soft rolls, gluten-free bread, cake, doughnuts, brioche, hamburger buns, Brussels wafels. In an aspect the baked products is a baked product comprising sucrose
5 such as bread containing from 2 to 30 wt% sugar based on total recipe weight, cake, doughnuts, brioche, hamburger buns, Brussels wafels. Gluten free bread herein and herein after is bread than contains at most 20 ppm gluten. Several grains and starch sources are considered acceptable for a gluten-free diet. Frequently used sources are potatoes, rice and tapioca (derived from cassava). A baked product herein includes
10 without limitation tin bread, loaves of bread, twists, buns, such as hamburger buns or steamed buns, chapati, rusk, dried steam bun slice, bread crumb, matzos, focaccia, melba toast, zwieback, croutons, soft pretzels, soft and hard bread, bread sticks, yeast leavened and chemically-leavened bread, laminated dough products such as Danish pastry, croissants or puff pastry products, muffins, danish, bagels, confectionery coatings,
15 crackers, wafers, pizza crusts, tortillas, pasta products, crepes, waffles, parbaked products. An example of a parbaked product includes, without limitation, partially baked bread that is completed at point of sale or consumption with a short second baking process. A baked product herein includes without limitation pound cake, butter cake, sponge cake, biscuit cake, roulade, genoise and chiffon cake, foam cakes.

20 The bread may be white or brown pan bread; such bread may for example be manufactured using a so called American style Sponge and Dough method or an American style Direct method.

 The term tortilla herein includes corn tortilla and wheat tortilla. A corn tortilla is a type of thin, flat bread, usually unleavened made from finely ground maize (usually
25 called "corn" in the United States). A flour tortilla is a type of thin, flat bread, usually unleavened, made from finely ground wheat flour. The term tortilla further includes a similar bread from South America called arepa, though arepas are typically much thicker than tortillas. The term tortilla further includes a laobing, a pizza-shaped thick "pancake" from China and an Indian Roti, which is made essentially from wheat flour. A tortilla
30 usually has a round or oval shape and may vary in diameter from about 6 to over 30 cm.

 In an embodiment of the process for the production of baked product the baked product is bread or cake.

The alpha-amylase variant according to the invention, the enzyme composition according to the invention or the premix according to the invention may be used in the preparation of a wide range of cakes, including shortened cakes, such as for example pound cake and butter cake, and including foam cakes, such as for example meringues, sponge cake, biscuit cake, roulade, genoise and chiffon cake. Sponge cake is a type of soft cake based on wheat flour, sugar, baking powder and eggs (and optionally baking powder). The only fat present is from the egg yolk, which is sometimes added separately from the white. It is often used as a base for other types of cakes and desserts. A pound cake is traditionally prepared from one pound each of flour, butter, eggs, and sugar, optionally complemented with baking powder. In chiffon cake the butter/margarine has been replaced by oil. Sugar and egg yolk is decreased compared to pound or sponge cake and egg white content is increased.

A method to prepare a batter preferably comprises the steps of:

- a. preparing the batter of the cake by adding at least:
 - i. sugar;
 - ii. flour;
 - iii. the alpha-amylase variant according to the invention;
 - iv. egg; and
 - v. optionally a phospholipase.

A method to prepare a cake according to the invention further comprises the step of

- b. baking the batter to yield a cake.

Alternatively a method to prepare a batter may comprise the steps of:

- a. preparing the batter of the cake by adding at least:
 - i. sugar;
 - ii. flour;
 - iii. the alpha-amylase variant according to the invention;
 - iv. fat; and
 - v. optionally a phospholipase.

A method to prepare a cake according to the invention further comprises the step of

- b. baking the batter to yield a cake.

The person skilled in the art knows how to prepare a batter or a cake starting from dough ingredients. Optionally one or more other ingredients can be present in the

composition e.g. to allow reduction of eggs and/or fat in the cake, such as hydrocolloids, yeast extract, non-egg proteins (including whey protein concentrate and soy protein concentrate), emulsifiers, calcium, one or more enzyme(s).

5 A cake batter according to the invention comprise the alpha-amylase variant according to the invention in an effective amount.

A cake batter according to the invention may contain based on total weight of the batter:

- 20-40 wt% flour (or combination of flour and wheat starch);
- 5-30 wt% egg;
- 15-35 wt% sugar; and
- 10 - 5-35 wt% fat.

A batter for making an egg-less cake according to the invention may contain based on total weight of the batter:

- 20-40 wt% flour (or combination of flour and wheat starch);
- 15-35 wt% sugar; and
- 15 - 10-35 wt% fat.

A batter for making a low fat cake according to the invention may contain based on total weight of the batter:

- 20-40 wt% flour(or combination of flour and wheat starch);
- 5-30 wt% egg;
- 20 - 15-35 wt% sugar; and
- 5-15 wt% fat.

A batter for making a pound cake according to the invention typically contains equal wt% by weight of the batter of each of flour, sugar, egg and fat.

25 A batter for making a sponge cake according to the invention may contain based on total weight of the batter:

- 25-35 wt% flour (or combination of flour and wheat starch);
- 15-40 wt% egg; and
- 20-35 wt % sugar.

30 A batter for making a high ratio cake according to the invention may contain based on total weight of the batter:

- 20-40 wt% flour (or combination of flour and wheat starch);
- 15-30 wt% egg;
- 20-35 wt% sugar; and
- 10-25 wt% fat.

In general in a high ratio cake recipe the amount of liquid and sugar exceeds the flour content.

A batter for making a muffin according to the invention may contain based on total weight of the batter:

- 5 - 20-40 wt% flour (or combination of flour and wheat starch);
- 5- 30 wt% egg;
- 10-35 wt% sugar; and
- 10-30 wt% fat (usually in the form of oil or melted butter / margarine).

10 The present invention also provides a baked product obtainable by the process according to the invention or by the use according to the invention.

 The invention relates to variant polypeptides having alpha-amylase activity, i.e. to alpha-amylase variants. An alpha-amylase variant of the invention may have one or more improved properties in comparison with a reference polypeptide, the reference
15 polypeptide typically having alpha-amylase activity (such as the polypeptide as set out in SEQ ID NO: 2).

 The improved property will typically be a property with relevance to the use of the variant alpha-amylase in the preparation of a baked product. An improved property is determined by comparison of the performance of the variant and the reference
20 polypeptide under the same conditions.

 The improved property may include:
one or more of

- a) an increased thermostability;
- b) an increased sucrose tolerance;
- 25 c) an increased thermostability in the presence of sucrose
- d) an increased Activity at pH4 : Activity at pH5 ratio;
- e) an increased Activity at pH7 : Activity at pH5 ratio; and
- f) an increased specific activity,
- as compared to a reference polypeptide having alpha-amylase activity(such as
30 the polypeptide of SEQ ID NO: 2).

 The improved property may include

g) an increased ratio of the activity at a temperature of 50 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference

polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

In an aspect the improved property includes an increased Activity at 50°C or higher (amount of substrate converted per minute) : Activity at 37°C (amount of substrate converted per minute) ratio of a variant according to the invention as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2). The amount of substrate converted per minute may be the amount of glucose released per minute.

The improved property may include:

h) an increased ratio of the activity at a temperature of 60 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

In an aspect the improved property may include an increased Activity at 60°C or higher (amount of substrate converted per minute) : Activity at 37°C (amount of substrate converted per minute) ratio of a variant according to the invention as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2). The amount of substrate converted per minute may be the amount of glucose released per minute.

The improved property may include:

i) an increased ratio of the activity at a temperature of 60 degrees Celsius versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

In an aspect the improved property may include an increased Activity at 60°C (amount of substrate converted per minute) : Activity at 37°C (amount of substrate converted per minute) ratio of a variant according to the invention as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The amount of substrate converted per minute may be the amount of glucose released per minute.

The improved property may include:

j) an increased ratio of the activity at a temperature of 80 degrees Celsius versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide

having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

In an aspect the improved property may include an increased Activity at 80°C (amount of substrate converted per minute) : Activity at 37°C (amount of substrate converted per minute) ratio of a variant according to the invention compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2).

The amount of substrate converted per minute may be the amount of glucose released per minute.

10 Sucrose tolerance

In an aspect of the invention determination of Sucrose Tolerance of a variant polypeptide is done via measuring the [Activity in the presence of sucrose] and the [Activity in the absence of sucrose] of both the variant polypeptide and the reference polypeptide.

15 The [Activity in the absence of sucrose] may be determined using Assay 2A as described in the Materials and Methods section herein.

Activity in the presence of sucrose is determined at a suitable pH, such as pH4, pH5, pH6 or pH7.

20 The [Activity in the presence of sucrose] may be determined using assay 2M (at pH 4), using assay 2B (at pH 5) or assay 2H (at pH 7) as described in the Materials and Methods section herein. The [Activity in the presence of sucrose] at pH 6 may be determined analogously.

25 Sucrose tolerance of a variant according to the invention may be expressed as the ratio of

[Activity of a variant in the presence of sucrose] to [Activity of the variant in the absence of sucrose],

expressed as a percentage of the ratio of

30 [Activity of a reference polypeptide having alpha-amylase activity in the presence of sucrose] to [Activity of the reference polypeptide having alpha-amylase activity in the absence of sucrose].

Sucrose tolerance of a variant according to the invention may be expressed as the ratio of

[Activity on maltotriose of a variant in the presence of sucrose] to [Activity on maltotriose of the variant in the absence of sucrose],

expressed as a percentage of the ratio of

[Activity on maltotriose of a reference polypeptide having alpha-amylase activity
5 in the presence of sucrose] to [Activity on maltotriose of the reference polypeptide having
alpha-amylase activity in the absence of sucrose].

In an aspect of sucrose tolerance of a variant according to the invention, the sucrose tolerance is the sucrose tolerance at pH 7. The sucrose tolerance at pH 7 may

10 be expressed as the ratio of

[Activity of the variant in Assay 2H (as described herein)] to [Activity of the variant in Assay 2A (as described herein)],

expressed as a percentage of the ratio of

[Activity of the reference polypeptide in Assay 2H (as described herein)] to [Activity of
15 the reference polypeptide in Assay 2A (as described herein)].

The percentage thus obtained may be used as measure for the sucrose tolerance of the variant according to the invention. A sucrose tolerance of more than 100% shows that the variant has an increased sucrose tolerance compared to the reference
20 polypeptide having alpha-amylase activity.

In an aspect of the invention the variant according to the invention has an increased sucrose tolerance compared with a reference polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase activity has
25 an amino acid sequence as set out in SEQ ID NO: 2.

In an aspect of the invention the variant according to the invention has an increased sucrose tolerance at pH 4 compared with a reference polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase activity has an amino acid sequence as set out in SEQ ID NO: 2.

30 In an aspect of the invention the variant according to the invention has an increased sucrose tolerance at pH 5 compared with a reference polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase activity has an amino acid sequence as set out in SEQ ID NO: 2.

In an aspect of the invention the variant according to the invention has an increased sucrose tolerance at pH 7 compared with a reference polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase activity has an amino acid sequence as set out in SEQ ID NO: 2.

5

Thermostability

Thermostability may be determined by measuring the residual activity after incubation at a higher temperature (e.g. 50-100°C for 1-30 min), using a suitable activity assay (such as Ceralpha) or alternatively the NBAU assay as described herein.

10 Thermostability may be determined using an assay described herein. Thermostability may be determined at a suitable pH such as at pH 4, at pH 5, pH 6 or pH7. Thermostability may be determined in the presence of sucrose.

In an aspect of the invention the determination of Thermostability of a variant polypeptide is done via measuring the [Residual Activity of a variant determined after an incubation at a temperature of above 37 degrees Celsius] and the [Activity after an incubation at a temperature of 37 degrees Celsius] of both the variant polypeptide and the reference polypeptide.

15 Thermostability of a variant according to the invention may be expressed as the ratio of
20 [Residual Activity of a variant determined after an incubation at a temperature of above 37 degrees Celsius] to [Activity of the variant determined after an incubation at a temperature of 37 degrees Celsius],
expressed as a percentage of the ratio of
[Residual Activity of a reference polypeptide having alpha-amylase activity after an incubation at a temperature of above 37 degrees Celsius] to [Activity of the reference polypeptide having alpha-amylase activity after an incubation at a temperature of 37 degrees Celsius].

25 In a further aspect the thermostability of a variant according to the invention is determined using maltotriose as a substrate.

30 The [Activity of the variant determined after an incubation at a temperature of 37 degrees Celsius], may be determined using Assay 2A as described in the Materials and Methods section herein.

The exposure to a temperature of above 37 degrees Celsius is done at a suitable pH, such as pH4, pH5, pH6 or pH7. After the incubation at a temperature of above 37 degrees Celsius at this pH, the residual activity is measured at pH 5 to yield the value for [Residual Activity of a variant determined after an incubation at a temperature of above 37 degrees Celsius] .

The [Residual Activity of a variant determined after an incubation at a temperature of above 37 degrees Celsius] may be determined using assay 2E (exposure to a temperature of above 37 degrees Celsius at pH 4), using assay 2C (exposure to a temperature of above 37 degrees Celsius at pH 5) or assay 2J (exposure to a temperature of above 37 degrees Celsius at pH 7) as described in the Materials and Methods section herein.

The Thermostability at pH 6 may be determined analogously.

In an aspect of Thermostability of a variant according to the invention, the thermostability is the thermostability at pH 5. The thermostability at pH 5 may be expressed as the ratio of

[Residual Activity of a variant determined after an incubation at a temperature of above 37 degrees Celsius at pH 5] to [Activity of the variant determined after an incubation at a temperature of 37 degrees Celsius at pH 5],

expressed as a percentage of the ratio of

[Residual Activity of a reference polypeptide having alpha-amylase activity after an incubation at a temperature of above 37 degrees Celsius at pH 5] to [Activity of the reference polypeptide having alpha-amylase activity after an incubation at a temperature of 37 degrees Celsius at pH 5].

In an aspect thermostability at pH 5 of a variant according to the invention may be expressed as the ratio of

[Activity of the variant in Assay 2C (as described herein)] to [Activity of the variant in Assay 2A (as described herein)],

expressed as a percentage of the ratio of

[Activity of the reference polypeptide in Assay 2C (as described herein)] to [Activity of the reference polypeptide in Assay 2A (as described herein)].

The percentage thus obtained may be used as measure for the thermostability at pH 5 of the variant according to the invention. A thermostability at pH 5 of more than

100% shows that the variant has an increased thermostability at pH 5 compared to the reference polypeptide having alpha-amylase activity.

Thermostability at pH 4, at pH 6 or t pH 7 of a variant polypeptide according to the invention can be determined analogously.

5 A thermostability of more than 100% shows that the variant has an increased thermostability compared to the reference polypeptide having alpha-amylase activity.

 In an aspect of the invention the variant polypeptide according to the invention has an increased thermostability at pH 4 compared with a reference polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase
10 activity has an amino acid sequence as set out in SEQ ID NO: 2.

 In an aspect of the invention the variant polypeptide according to the invention has an increased thermostability at pH 5 compared with a reference polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase activity has an amino acid sequence as set out in SEQ ID NO: 2.

15 In an aspect of the invention the variant according to the invention has an increased thermostability at pH 7 compared with a reference polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase activity has an amino acid sequence as set out in SEQ ID NO: 2.

20 Thermostability in the presence of sucrose

 In an aspect of the invention the determination of Thermostability in the presence of sucrose of a variant polypeptide is done via measuring the [Residual Activity of a variant determined after an incubation in the presence of sucrose at a temperature of above 37 degrees Celsius] and the [Activity after an incubation at a temperature of 37
25 degrees Celsius in the absence of sucrose] of both the variant polypeptide and the reference polypeptide.

 Thermostability in the presence of sucrose of a variant according to the invention may be expressed as the ratio of
[Residual Activity of a variant determined after incubation in the presence of sucrose at a
30 temperature of above 37 degrees Celsius] to [Activity of the variant determined after incubation in the absence of sucrose at a temperature of 37 degrees Celsius],
expressed as a percentage of the ratio of
[Residual Activity of a reference polypeptide having alpha-amylase activity determined after incubation in the presence of sucrose at a temperature of above 37 degrees

Celsius] to [Activity of the reference polypeptide having alpha-amylase activity determined after incubation in the absence of sucrose at a temperature of 37 degrees Celsius].

5 In a further aspect the thermostability in the presence of sucrose of a variant according to the invention is determined using maltotriose as a substrate.

The [Activity of the variant determined after incubation in the absence of sucrose at a temperature of 37 degrees Celsius], may be determined using Assay 2A as described in the Materials and Methods section herein.

10 The exposure to a temperature of above 37 degrees Celsius in the presence of sucrose is done at a suitable pH, such as pH4, pH5, pH6 or pH7. After the incubation in the presence of sucrose at a temperature of above 37 degrees Celsius at this pH, the residual activity is measured at pH 5 to yield the value for [Residual Activity of a variant determined after an incubation in the presence of sucrose at a temperature of above 37
15 degrees Celsius] .

The [Residual Activity of a variant determined after an incubation in the presence of sucrose at a temperature of above 37 degrees Celsius] may be determined using assay 2N (exposure to a temperature of above 37 degrees Celsius at pH 4), using assay 2F (exposure to a temperature of above 37 degrees Celsius at pH 5) or assay 2K
20 (exposure to a temperature of above 37 degrees Celsius at pH 7) as described in the Materials and Methods section herein.

The Thermostability in the presence of sucrose at pH 6 may be determined analogously.

Thermostability in the presence of sucrose of a variant according to the invention may be expressed as the ratio of

[Residual Activity on maltotriose of a variant determined after incubation in the presence of sucrose at a temperature of above 37 degrees Celsius] to [Activity on maltotriose of the variant determined after incubation in the absence of sucrose at a temperature of 37 degrees Celsius],

expressed as a percentage of the ratio of

[Residual Activity on maltotriose of a reference polypeptide having alpha-amylase activity determined after incubation in the presence of sucrose at a temperature of above 37 degrees Celsius] to [Activity on maltotriose of the reference polypeptide having alpha-amylase activity determined after incubation in the absence of sucrose at a temperature of 37 degrees Celsius].

In an aspect of thermostability in the presence of sucrose of a variant according to the invention, the thermostability in the presence of sucrose is the thermostability in the presence of sucrose at pH 5. The thermostability in the presence of sucrose at pH 5 may be expressed as the ratio of

[Residual Activity of the variant in Assay 2F (as described herein)] to [Activity on of the variant in Assay 2A (as described herein)],

expressed as a percentage of the ratio of

[Residual Activity on maltotriose of the reference polypeptide in Assay 2F (as described herein)] to [Activity on maltotriose of the reference polypeptide in Assay 2A (as described herein)].

The percentage thus obtained may be used as measure for the thermostability in the presence of sucrose of the variant according to the invention. A thermostability in the presence of sucrose of more than 100% shows that the variant has an increased thermostability in the presence of sucrose compared to the reference polypeptide having alpha-amylase activity.

In an aspect of the invention the variant according to the invention has an increased thermostability in the presence of sucrose compared with a reference polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase activity has an amino acid sequence as set out in SEQ ID NO: 2.

In an aspect of the invention the variant according to the invention has an increased thermostability in the presence of sucrose at pH 4 compared with a reference

polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase activity has an amino acid sequence as set out in SEQ ID NO: 2.

In an aspect of the invention the variant according to the invention has an increased thermostability in the presence of sucrose at pH 5 compared with a reference polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase activity has an amino acid sequence as set out in SEQ ID NO: 2.

In an aspect of the invention the variant according to the invention has an increased thermostability in the presence of sucrose at pH 7 compared with a reference polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase activity has an amino acid sequence as set out in SEQ ID NO: 2.

Activity at 50°C or higher : Activity at 37°C ratio

The “Activity at 50°C or higher : Activity at 37°C ratio” may also be referred to herein as “activity at a temperature of 50 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius”.

The Activity at 50°C or higher : Activity at 37°C ratio may be determined as the Activity at 60°C : Activity at 37°C ratio as described herein.

The Activity at 50°C or higher : Activity at 37°C ratio may be determined as the Activity at 80°C : Activity at 37°C ratio as described herein.

In an aspect of the invention the determination of the Activity at 50°C or higher : Activity at 37°C ratio of a variant polypeptide is done via measuring the [Activity of a variant determined during an incubation at a temperature of above 50 degrees Celsius or higher] and the [Activity during an incubation at a temperature of 37 degrees Celsius] of both the variant polypeptide and the reference polypeptide.

The Activity at 50°C or higher : Activity at 37°C ratio of a variant according to the invention may be expressed as the ratio of

[Activity of a variant determined during an incubation at a temperature of 50 degrees Celsius or higher] to [Activity of the variant determined during an incubation at a temperature of 37 degrees Celsius],

expressed as a percentage of the ratio of

- 5 [Activity of a reference polypeptide having alpha-amylase activity during an incubation at a temperature of 50 degrees Celsius or higher] to [Activity of the reference polypeptide having alpha-amylase activity during an incubation at a temperature of 37 degrees Celsius].

- 10 In a further aspect the Activity at 50°C or higher : Activity at 37°C ratio of a variant according to the invention is determined using maltotriose as a substrate.

The [Activity of the variant determined during an incubation at a temperature of 37 degrees Celsius], may be determined using Assay 2A as described in the Materials and Methods section herein.

- 15 The [Activity of the variant determined during an incubation at a temperature of 50 degrees Celsius or higher], may be determined using Assay 2P or 2Q as described in the Materials and Methods section herein or an assay applied analogously to the desired incubation temperature.

Activity at 60°C : Activity at 37°C ratio

- 20 Activity at 60°C (amount of substrate converted per minute) of variants according to the invention may be determined using Assay 2P as described herein.

Activity at 37°C (amount of substrate converted per minute) of variants according to the invention may be determined using Assay 2A as described herein.

- 25 Activity at 60°C (amount of substrate converted per minute) : Activity at 37°C (amount of substrate converted per minute) ratio of variants according to the invention may be expressed as the ratio of:

[Activity of the variant in Assay 2P (as described herein)] to [Activity of the variant in Assay 2A (as described herein)],

expressed as a percentage of the ratio of

- 30 [Activity of the reference polypeptide in Assay 2P (as described herein)] to [Activity of the reference polypeptide in Assay 2A (as described herein)].

An Activity at 60°C : Activity at 37°C ratio of more than 100% shows that the variant has an increased Activity at 60°C : Activity at 37°C compared to the reference polypeptide.

In an aspect of the invention the variant according to the invention has an increased Activity at 60°C : Activity at 37°C ratio compared with a reference polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase activity has an amino acid sequence as set out in SEQ ID NO: 2.

Activity 80°C : Activity at 37°C ratio

Activity at 80°C (amount of substrate converted per minute) of variants according to the invention may be determined using Assay 2Q as described herein.

Activity at 37°C (amount of substrate converted per minute) of variants according to the invention may be determined using Assay 2A as described herein.

Activity at 80°C (amount of substrate converted per minute) : Activity at 37°C (amount of substrate converted per minute) ratio of variants according to the invention may be expressed as the ratio of:

[Activity of the variant in Assay 2Q (as described herein)] to [Activity of the variant in Assay 2A (as described herein)],

expressed as a percentage of the ratio of

[Activity of the reference polypeptide in Assay 2Q (as described herein)] to [Activity of the reference polypeptide in Assay 2A (as described herein)].

An Activity at 80°C : Activity at 37°C ratio of more than 100% shows that the variant has an increased the Activity at 80°C : Activity at 37°C compared to the reference polypeptide.

In an aspect of the invention the variant according to the invention has an increased Activity at 80°C : Activity at 37°C ratio compared with a reference polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase activity has an amino acid sequence as set out in SEQ ID NO: 2.

Activity at pH4 : Activity at pH5 ratio

Activity at pH 4 and Activity at pH 5 may be determined using a suitable assay such as the Maltotriose assay as described herein and adjusting the pH accordingly.

Activity at pH4 : Activity at pH5 ratio of a variant according to the invention may be expressed as the ratio of
[Activity of a variant determined at pH 4] to [Activity of the variant determined at pH 5],
expressed as a percentage of the ratio of
[Activity of a reference polypeptide at pH 4] to [Activity of the reference polypeptide at a pH 5].

10

Activity at pH4 : Activity at pH5 ratio of a variant according to the invention may be expressed as the ratio of
[Activity on maltotriose of a variant determined at pH 4] to [Activity on maltotriose of the variant determined at pH 5],

15

expressed as a percentage of the ratio of
[Activity on maltotriose of a reference polypeptide at pH 4] to [Activity on maltotriose of the reference polypeptide at pH 5].

20

In an aspect Activity at pH4 : Activity at pH5 ratio of a variant according to the invention may be expressed as the ratio of
[Activity of the variant in Assay 2D (as described herein)] to [Activity of the variant in Assay 2A (as described herein)],
expressed as a percentage of the ratio of
[Activity on maltotriose of the reference polypeptide in Assay 2D (as described herein)] to [Activity on maltotriose of the reference polypeptide in Assay 2A (as described herein)].

25

The percentage thus obtained may be used as measure for the Activity at pH4 : Activity at pH5 ratio of the variant according to the invention. An Activity at pH4 : Activity at pH5 ratio of more than 100% shows that the variant has an increased Activity at pH4 : Activity at pH5 ratio compared to the reference polypeptide having alpha-amylase activity.

30

An Activity at pH4 : Activity at pH5 ratio of more than 100% may be used to demonstrate an altered pH profile in comparison with a parent polypeptide having alpha-amylase activity, for example to be more suitable for sour dough or other lower pH baking applications.

In an aspect of the invention the variant according to the invention has an increased Activity at pH4 : Activity at pH5 ratio compared with a reference polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase activity has an amino acid sequence as set out in SEQ ID NO: 2.

5

Activity at pH7 : Activity at pH5 ratio

The Activity at pH7 : Activity at pH5 ratio may be determined analogously as described above for Activity at pH4 : Activity at pH5 ratio .

The percentage thus obtained may be used as measure for the Activity at pH7 : Activity at pH5 ratio of the variant according to the invention. An Activity at pH7 : Activity at pH5 ratio of more than 100% shows that the variant has an increased Activity at pH7 : Activity at pH5 ratio compared to the reference polypeptide having alpha-amylase activity.

An Activity at pH7 : Activity at pH5 ratio of more than 100% may be used to demonstrate an altered pH profile in comparison with a parent polypeptide having alpha-amylase activity for example to be more suitable for cake or other higher pH baking applications.

In an aspect of the invention the variant according to the invention has an increased Activity at pH7 : Activity at pH5 ratio compared with a reference polypeptide having alpha-amylase activity, preferably the reference polypeptide having alpha-amylase activity has an amino acid sequence as set out in SEQ ID NO: 2.

Specific Activity

Specific Activity may be determined by measuring the activity per mg of protein (amount of protein can e.g. be estimated from SDS-PAGE, or if sample is pure enough can be determined using Bradford assay). Suitable examples of an assay to determine the specific activity include the NBAU assay and the Maltotriose assay as described herein.

A way to determine the specific activity is to determine the [Activity of (a sample containing) the variant] and also determine the protein content of such sample.

In an aspect the Specific Activity is expressed as [Activity of a variant at pH X] in U/ml divided by the Protein content in mg/ml.

In a further aspect the specific activity is determined using maltotriose as a substrate.

In an aspect the Specific Activity is determined using maltotriose as a substrate and is expressed as [Activity of a variant at pH X] in U/ml divided by the Protein content in mg/ml.

5 The [Activity of the variant at pH4], may be determined using Assay 2D as described in the Materials and Methods section herein.

The [Activity of the variant at pH5], may be determined using Assay 2A as described in the Materials and Methods section herein.

The [Activity of the variant at pH7], may be determined using Assay 2G as described in the Materials and Methods section herein.

10 The Specific Activity of the variant at pH6 may be determined analogously.

The Protein content may be determined using the Bradford assay as described in Assay 2L herein in the Materials and Methods section.

15 Activity at different pH (determine pH optimum) may be determined by measuring the activity in a pH range (e.g. 2-12).

pH stability may be determined by measuring the thermostability in a pH range (measure thermostability as described above, but do incubation in a range of pH values, e.g. 2-12).

20 Activity on raw starch may be determined by incubating the enzyme with a suspension of native starch (e.g. wheat or maize), followed by centrifuging to remove starch granules, and determining the amount of reducing sugars released (e.g. with DNS (dinitrosalicylic) method).

25 Altered temperature optimum may be determined by measuring activity as described above over a temperature range (e.g. 50-100°C)

Substrate specificity may be determined by measuring activity as described above on different substrates.

In one embodiment an alpha-amylase variant according to the invention may have a pH optimum which is altered compared to the parent polypeptide

30 In one embodiment an alpha-amylase variant according to the invention may have a pH optimum which is higher than that of the parent polypeptide having alpha-amylase activity or lower than such parent polypeptide.

Preferably the parent polypeptide is that according to SEQ ID NO: 2. For example, the wild-type alpha-amylase from *Alicyclobacillus pohliae* (as disclosed in SEQ ID NO: 2) has a pH optimum of from pH 4 to pH 5.

Preferably the parent polypeptide is that according to SEQ ID NO: 2. For example, the
5 wild-type alpha-amylase from *Alicyclobacillus pohliae* (as disclosed in SEQ ID NO: 2) has a pH optimum of from pH 4,5 to pH 5,5. In an aspect an alpha-amylase variant of the invention may be more alkaliphilic than such a wild-type enzyme, i.e. may, for example, have a pH optimum of from pH 5 to pH 8, preferably from pH 6 to pH 7. Optionally a variant protein of the invention may be more acidophilic than the wild type alpha-amylase.

10

In an aspect an alpha-amylase variant according to the invention may have a specific activity which is higher than that of the parent polypeptide measured at the same pH. With specific activity of a variant protein it is herewith intended the alpha amylase activity of the alpha-amylase variant measured in units/mg of pure protein. Preferably the
15 specific activity of the alpha-amylase variant according to the invention is higher at at least one pH, preferably a pH between 4 and 8, than that of the parent polypeptide measured at the same pH.

As indicated above the improved property will typically be a property with
20 relevance to the use of the variant alpha-amylase in the preparation of a baked product.

The term "improved property" includes without limitation any property of a dough and/or a product obtained from the dough, particularly a baked product, which is improved by the action of the alpha-amylase variant, the composition according to the invention or the pre-mix according to the invention relative to a dough or product in which
25 a parent polypeptide is incorporated. The improved property may include, but is not limited to, increased strength of the dough, increased elasticity of the dough, increased stability of the dough, reduced stickiness of the dough, improved extensibility of the dough, improved machineability of the dough, increased volume of the baked product, improved flavour of the baked product, improved crumb structure of the baked product,
30 improved crumb softness of the baked product, reduced blistering of the baked product, improved crispiness, improved resilience both initial and in particular after storage, reduced hardness after storage and/or improved anti-staling of the baked product.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant provides improved anti-staling to a baked product compared with a reference polypeptide (such as the polypeptide as set out in SEQ ID NO: 2).

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant provides improved anti-staling to a baked product compared with a reference polypeptide (such as the polypeptide as set out in SEQ ID NO: 2).

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant provides improved anti-staling to a baked product compared with a reference polypeptide (such as the polypeptide as set out in SEQ ID NO: 2).

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant provides a further reduced hardness after storage to a baked product compared with compared with a reference polypeptide having alpha amylase activity (such as the polypeptide of SEQ ID NO: 2).

5 In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to
10 any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant provides a further reduced hardness after storage to a baked product compared with compared with a reference polypeptide having alpha amylase activity (such as the polypeptide of SEQ ID NO: 2).

15 In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to
20 any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant provides a further reduced hardness after storage to a baked product compared with compared with a reference polypeptide having alpha amylase activity (such as the polypeptide of SEQ ID NO: 2).

25

 In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y)
30 and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant provides a further decreased loss of resilience after storage to a baked product compared with compared with a reference polypeptide having alpha

amylase activity compared with a reference polypeptide having alpha amylase activity (such as the polypeptide of SEQ ID NO: 2).

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue
5 corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

10 and wherein the variant provides a further decreased loss of resilience after storage to a baked product compared with compared with a reference polypeptide having alpha amylase activity compared with a reference polypeptide having alpha amylase activity (such as the polypeptide of SEQ ID NO: 2).

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue
15 corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

20 and wherein the variant provides a further decreased loss of resilience after storage to a baked product compared with compared with a reference polypeptide having alpha amylase activity compared with a reference polypeptide having alpha amylase activity (such as the polypeptide of SEQ ID NO: 2).

25

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y),
30 and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant provides a further reduced hardness after storage to a baked product, said baked product comprising at least 2 wt% sugar based on total recipe

weight compared with a reference polypeptide having alpha amylase activity (such as the polypeptide of SEQ ID NO: 2). In a further aspect of this embodiment the baked product comprises at least 3 wt% sugar, in an aspect comprising at least 5 wt% sugar, in an aspect at least 8 wt% sugar, in an aspect at least 12 wt% sugar, in an aspect at least 15 wt% sugar based on total recipe weight.

In a further aspect of this embodiment the baked product comprises at least 18 wt% sugar, in an aspect at least 20 wt% sugar, in an aspect at least 25 wt% sugar, in an aspect at least 30 wt% sugar based on total recipe weight.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant provides a further reduced hardness after storage to a baked product, said baked product comprising at least 2 wt% sugar based on total recipe weight compared with a reference polypeptide having alpha amylase activity (such as the polypeptide of SEQ ID NO: 2).

In a further aspect of this embodiment the baked product comprises at least 3 wt% sugar, in an aspect at least 5 wt% sugar, in an aspect at least 8 wt% sugar, in an aspect at least 12 wt% sugar, in an aspect at least 15 wt% sugar based on total recipe weight. In a further aspect of this embodiment the baked product comprises at least 18 wt% sugar, in an aspect at least 20 wt% sugar, in an aspect at least 25 wt% sugar, in an aspect at least 30 wt% sugar based on total recipe weight.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant provides a further reduced hardness after storage to a baked product, said baked product comprising at least 5 wt% sugar based on total recipe

weight compared with a reference polypeptide having alpha amylase activity (such as the polypeptide of SEQ ID NO: 2).

In a further aspect of this embodiment the baked product comprises at least 8 wt% sugar, in an aspect at least 12 wt% sugar, in an aspect at least 15 wt% sugar based on total recipe weight. In a further aspect of this embodiment the baked product comprises at
5 least 18 wt% sugar, in an aspect at least 20 wt% sugar, in an aspect at least 25 wt% sugar, in an aspect at least 30 wt% sugar based on total recipe weight.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the
10 sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 (preferably comprises the amino acid substitution W70Y) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

15 and wherein the variant provides a further decreased loss of resilience to a baked product, said baked product comprising at least 2 wt% sugar based on total recipe weight compared with a reference polypeptide having alpha amylase activity (such as the polypeptide of SEQ ID NO: 2).

In a further aspect of this embodiment the baked product comprises at least 3 wt% sugar,
20 in an aspect at least 5 wt% sugar, in an aspect at least 8 wt% sugar, in an aspect at least 12 wt% sugar, in an aspect at least 15 wt% sugar based on total recipe weight. In a further aspect of this embodiment the baked product comprises at least 18 wt% sugar, in an aspect at least 20 wt% sugar, in an aspect at least 25 wt% sugar, in an aspect at least 30 wt% sugar based on total recipe weight.

25 In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 (preferably comprises the amino acid substitution L225F), and at least one further substitution of an amino acid residue corresponding to
30 any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant provides a further decreased loss of resilience to a baked product, said baked product comprising at least 2 wt% sugar based on total recipe

weight compared with a reference polypeptide having alpha amylase activity (such as the polypeptide of SEQ ID NO: 2).

In a further aspect of this embodiment the baked product comprises at least 3 wt% sugar, in an aspect at least 5 wt% sugar, in an aspect at least 8 wt% sugar, in an aspect at least 12 wt% sugar, in an aspect at least 15 wt% sugar based on total recipe weight. In a further aspect of this embodiment the baked product comprises at least 18 wt% sugar, in an aspect at least 20 wt% sugar, in an aspect at least 25 wt% sugar, in an aspect at least 30 wt% sugar based on total recipe weight.

In an embodiment of the alpha-amylase polypeptide variant according to the invention, said variant has an amino acid sequence which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 (preferably comprises the amino acid substitution S200N) and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2;

and wherein the variant provides a further decreased loss of resilience to a baked product, said baked product comprising at least 2 wt% sugar based on total recipe weight compared with a reference polypeptide having alpha amylase activity (such as the polypeptide of SEQ ID NO: 2).

In a further aspect of this embodiment the baked product comprises at least 3 wt% sugar, in an aspect at least 5 wt% sugar, in an aspect at least 8 wt% sugar, in an aspect at least 12 wt% sugar, in an aspect at least 15 wt% sugar based on total recipe weight. In a further aspect of this embodiment the baked product comprises at least 18 wt% sugar, in an aspect at least 20 wt% sugar, in an aspect at least 25 wt% sugar, in an aspect at least 30 wt% sugar based on total recipe weight.

Improved mouth feel includes sense of softness on an initial bite or after chewing, preferably without a sticky feeling in the mouth and/or without the baked product sticking to the teeth. Improved mouth feel includes the baked product feeling less dry in the mouth on an initial bite or after chewing. Improved mouth feel includes the baked product feeling less dry in the mouth on an initial bite or after chewing after it has been kept outside its packaging or container. The improved property may include that after a slice of bread was taken from its packaging or container and exposed to ambient conditions

for 5 minutes, in an aspect for 10 minutes, in an aspect for 20 minutes it has improved mouthfeel.

The improved property may include that after a the cookie was taken from its packaging or container and exposed to ambient conditions for 10 minutes, in an aspect for 20
5 minutes, in an aspect for 30 minutes, in an aspect an hour it has improved mouthfeel. In an aspect ambient conditions herein and herein after include a temperature of 20 degrees C and a moisture level of 40 % humidity.

Reduced breaking during transport includes the baked product , including without limitation cookies, bread such as gluten free bread, does not break in additional pieces
10 as a consequence of transport.

Improved softness on squeeze includes the tactile experience that if a bun is held between the fingers and the thumb of a hand and the thumb and fingers are moved towards each other it takes less force.

Improved foldability of a baked product may be determined as follows.

15 The baked product is laid on a flat surface. The baked product is folded by picking up one edge of the product and placing it on the opposite edge of the product. This way a folded baked product is obtained having a bend curve in an area located at or close to the center. The surface of the outside of the bend of folded baked product is visually inspected. The foldability is improved if fewer cracks are observed at or close to the
20 bend. This may be a particularly useful property if the baked product is a tortilla and/or a slice of bread.

Improved stackability may be determined as follows.

10 baked products are stacked on top of each other and sealed in a polymer package, such as polyethylene foil. This yields a pack of baked products. 10 packs of baked
25 product are stacked on top of each other and kept under ambient conditions for 3 days, in an aspect for 5 days in an aspect for 1 week, in an aspect for 2 weeks. Ambient conditions are conditions as defined herein. After this period the bottom pack of baked products is opened, the baked products are separated from each other and the surfaces of the products are visually inspected. The stackability is improved if less surface
30 damage is observed. Surface damage may be caused e.g. by rupture of the surface during separation of two baked products that were stacked on top of each other. . This may be a particularly useful property if the baked product is a tortilla.

Faster dough development time may be determined as follows

Dough development time is the time the dough need to reach maximum consistency, maximum viscosity before gluten strands begin to break down. It may be determined by measuring peak time, using a Farinograph® from Brabender®, Germany. If a faronigraph is used to determine dough development time, dough development time is
5 the time between the moment water is added and the moment the curve reaches its highest point. Peak time is preferably expressed in minutes.

Reduced dough stickiness may be determined as follows.

Dough stickiness is preferably determined on two separate batches of at least 8 dough pieces, with the Texture Analyser TAXT2i (Stable Micro Systems Ltd., Surrey,
10 UK) equipped with a 5 kg load cell in the measure force in compression mode with a cylindrical probe (25 mm diameter). Using pre- and post-test speeds of 2.0 mm/s, while the test speed is 1.0 mm/s. Dough pieces are centered and compressed 50% and the probe is held for 10 s at maximum compression. A negative peak value indicates dough stickiness. A less negative peak value indicates reduced dough stickiness.

15 Increased flexibility may be determined as follows.

The baked product is laid on a flat surface. The baked product is rolled to a shape similar to a pipe, this way a rolled baked product is obtained. The flexibility is improved if the rolled baked product remains its rolled up shape and does not roll open. This may be a particularly useful property if the baked product is a tortilla or a pancake.

20

The improved property may be determined by comparison of a dough and/or a baked product prepared with and without addition of the (isolated) polypeptide of the present invention in accordance with the methods of present invention which are described below in the Examples. Organoleptic qualities may be evaluated using
25 procedures well established in the baking industry, and may include, for example, the use of a panel of trained taste-testers.

The term “increased strength of the dough” is defined herein as the property of a dough that has generally more elastic properties and/or requires more work input to mould and shape.

30 The term “increased elasticity of the dough” is defined herein as the property of a dough which has a higher tendency to regain its original shape after being subjected to a certain physical strain.

The term “increased stability of the dough” is defined herein as the property of a dough that is less susceptible to forming faults as a consequence of mechanical abuse

thus better maintaining its shape and volume and is evaluated by the ratio of height: width of a cross section of a loaf after normal and/or extended proof.

The term "reduced stickiness of the dough" is defined herein as the property of a dough that has less tendency to adhere to surfaces, e.g., in the dough production machinery, and is either evaluated empirically by the skilled test baker or measured by
5 the use of a texture analyser (e.g. a TAXT Plus) as known in the art.

The term "improved extensibility of the dough" is defined herein as the property of a dough that can be subjected to increased strain or stretching without rupture.

The term "improved machineability of the dough" is defined herein as the property of a dough that is generally less sticky and/or more firm and/or more elastic.
10 Consequently there is less fouling of plant equipment and a reduced need for cleaning.

The term "increased volume of the baked product" is preferably measured as the volume of a given loaf of bread determined by an automated bread volume analyser (eg. BVM-3, TexVol Instruments AB, Viken, Sweden), using ultrasound or laser detection
15 as known in the art. In case the volume is increased, the property is improved. Alternatively the height of the baked product after baking in the same size tin is an indication of the baked product volume. In case the height of the baked product has increased, the volume of the baked product has increased.

The term "reduced blistering of the baked product" is defined herein as a visually
20 determined reduction of blistering on the crust of the baked bread.

The term "improved crumb structure of the baked product" is defined herein as the property of a baked product with finer cells and/or thinner cell walls in the crumb and/or more uniform/homogenous distribution of cells in the crumb and is usually evaluated visually by the baker or by digital image analysis as known in the art (eg. C-cell, Calibre Control International Ltd, Appleton, Warrington, UK).
25

The term "improved softness of the baked product" is the opposite of "hardness" and is defined herein as the property of a baked product that is more easily compressed and is evaluated either empirically by the skilled test baker or measured by the use of a texture analyzer (e.g. TAXT Plus) as known in the art. "hardness" may suitably be
30 evaluated either empirically by the skilled test baker or measured by the use of a texture analyzer (e.g. TAXT Plus) as known in the art.

"Resilience" of a baked product may be measured by the use of a texture analyzer (e.g. TAXT Plus) as known in the art.

“Improved cohesiveness of the baked” product may be demonstrated by measurement via the use of a texture analyser (e.g. TAXT Plus) as known in the art. If a texture analyser is used, cohesiveness is how well the product withstands a second deformation relative to how it behaved under the first deformation. It is measured as the area of work during the second compression divided by the area of work during the first compression. Cohesiveness may be used to evaluate eating / chewing behavior of the baked product.

The term “improved flavor of the baked product” is evaluated by a trained test panel.

The term “improved anti-staling of the baked product” is defined herein as the properties of a baked product that have a reduced rate of deterioration of quality parameters, e.g. reduced hardness after storage and/or decreased loss of resilience after storage.

Anti-staling properties may be demonstrated by a reduced hardness after storage of the baked product. The enzyme composition according to the invention or the pre-mix according to the invention may result in reduced hardness, e.g. in a baked product that is more easily compressed. The hardness of the baked product may be evaluated either empirically by the skilled test baker or measured by the use of a texture analyzer (e.g. TAXT Plus) as known in the art. The hardness measured within 24 hours after baking is called initial hardness. The hardness measured 24 hours or more after baking is called hardness after storage, and is also a measure for determining shelf life. In case the initial hardness has reduced, it has improved. In case the hardness after storage has reduced, it has improved. Preferably hardness is measured as described in example 9 herein.

Resilience of the baked product is preferably measured by the use of a texture analyzer (e.g. TAXTPlus) as known in the art.

The resilience measured within 24 hours after baking is called initial resilience. The resilience measured 24 hours or more after baking is called resilience after storage, and is also a measure for determining shelf life. Freshly baked product typically gives crumb of high initial resilience but resilience is lost over shelf-life. Improved anti-staling properties may be demonstrated by a reduced loss of resilience over storage. Preferably resilience is measured as described in example 9 herein .

The term “improved crispiness” is defined herein as the property of a baked product to give a crispier sensation than a reference product as known in the art, as well as to maintain this crispier perception for a longer time than a reference product. This

property can be quantified by measuring a force versus distance curve at a fixed speed in a compression experiment using e.g. a texture analyzer TA-XT Plus (Stable Micro Systems Ltd, Surrey, UK), and obtaining physical parameters from this compression curve, viz. (i) force of the first peak, (ii) distance of the first peak, (iii) the initial slope, (iv) the force of the highest peak, (v) the area under the graph and (vi) the amount of fracture events (force drops larger than a certain preset value). Indications of improved crispness are a higher force of the first peak, a shorter distance of the first peak, a higher initial slope, a higher force of the highest peak, higher area under the graph and a larger number of fracture events. A crispier product should score statistically significantly better on at least two of these parameters as compared to a reference product. In the art, "crispiness" is also referred to as crispness, crunchiness or crustiness, meaning a material with a crispy, crunchy or crusty fracture behaviour.

The volume increase of a baked product during the initial stage of baking is usually referred to as oven spring. Oven spring may be evaluated by determining the volume increase of a baked product during baking. Such volume increase may for example be determined by measuring the volume of the (fermented) dough before baking and subtracting the volume of the baked product (i.e. the volume of the dough after baking). A larger volume increase of a baked product produced with the variant polypeptide according to the invention as compared to the volume of baked product produced with a reference polypeptide having alpha-amylase activity indicates an improved oven spring.

Oven spring may be evaluated by visual assessment of a trained baker of the shape of the baked product such as bread. For example oven spring of a baked product may be assessed by visually judging the result after baking of an incision made in the dough before the dough entered the oven to be baked. For example by appointing a value in the range Oven spring: 1 = incision closed completely to 5 = completely open incision; teared. A higher value for a baked product produced with the variant polypeptide according to the invention as compared to baked product produced with a reference polypeptide having alpha-amylase activity indicates an improved oven spring. Oven spring may be evaluated by as a combination of volume increase during baking and the visual assessment of a trained baker on the shape of the baked product.

In industry baked products are often sliced. During the course of the slicing process of a baked product a deposit of a sticky layer may occur on the knives of the slicer. If this occurs this may lead to problems in plant bakeries. For example (big) holes

may appear in the baked product during slicing, this way causing damage to the baked product. This is not desirable. Slice-ability is therefore a desirable attribute of a baked product. Slice-ability may be determined of baked product having an elevated temperature, e.g a baked product that has after baking not completely cooled down to room temperature. A baked product at elevated temperature may have a temperature (as measured inside the bread) in a range of 40 to 50 degrees Celsius, such as a temperature of 46 to 48 degrees Celsius.

Slice-ability may be determined by slicing a baked product, such as bread, and visually inspecting the deposit on the knife or knives of the slicer. Slice-ability is improved if the amount of deposit on the knife or knives of the slicer used to slice a baked product produced with the variant polypeptide according to the invention is less as compared to the deposit on the knife or knives used to slice a baked product produced with a reference polypeptide having alpha-amylase.

Alternatively slice-ability may be determined by slicing a baked product and visually judging the slice. For example by appointing a value in the range Slice-ability: 1 = slice deformed, holes in the slice to 5 = slice not deformed, no holes in the slice. A higher value for a baked product produced with the variant polypeptide according to the invention as compared to baked product produced with a reference polypeptide having alpha-amylase activity indicates an improved slice-ability.

After slicing of a baked product, such as bread, slices may stick to each other, this is referred to herein as slice-stickiness. This may become apparent if one tries to separate bread slices within 1 to 2 hours, preferably 1 to 1,5 hours after the baked product was sliced and wrapped in a plastic bag. This may become apparent if one tries to separate bread slices when frozen. Herein slice-ability is improved if the tendency of slices to stick together of a sliced baked product produced with the variant polypeptide according to the invention is less as compared to the tendency of slices to stick together of a sliced baked product produced with a reference polypeptide having alpha-amylase. This may be determined manually by taking two adjacent slices and grasp one rim in each hand and pull the slices apart. If less force is needed to pull the slices apart from each other and/or when the slice is less damaged after pulling the slices apart, the slice-ability is improved.

Crumbliness of a baked product may be determined by slicing a baked product and weighing the amount of crumbs that are created. A lower weight of crumbs after slicing of a baked product produced with the variant polypeptide according to the

invention as compared to baked product produced with a reference polypeptide having alpha-amylase activity indicates an improved crumbliness.

The present invention may provide a dough having at least one of the improved properties selected from the group consisting of increased strength, increased elasticity,
5 increased stability, reduced stickiness, and/or improved extensibility of the dough.

The invention also may provide a baked product having increased loaf volume. The invention may provide as well a baked product having at least one improved property selected from the group consisting of increased volume, improved flavour, improved crumb structure, improved crumb softness, improved crispiness, reduced
10 blistering and/or improved anti-staling.

In aspect 1 the present invention includes a variant polypeptide according to the invention has alpha-amylase activity and has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2,
15 comprises a substitution of an amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue corresponding to any of amino acids

14, 15, 124, 133, 225, 200, 282, 358,

said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has
20 one or more altered properties as compared with a reference polypeptide having alpha-amylase activity.

In aspect 2 the present invention includes the variant according to aspect 1, wherein the variant has an amino acid sequence which comprises the amino acid substitution W70Y and at least one further substitution of an amino acid residue corresponding to any of
25 amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2.

In aspect 3 the present invention includes the variant according to aspect 2, wherein the variant has an amino acid sequence which comprises the amino acid substitution W70Y and one or more of

30 I14V, I15V, V124I, S133T, S200N, L225F, L282I, L282F and S358A, said positions being defined with reference to SEQ ID NO: 2.

In aspect 4 the present invention includes the variant according to aspect 1, wherein the variant has an amino acid sequence which comprises a substitution of the amino acid

residues corresponding to amino acids 70 and 200, said positions being defined with reference to SEQ ID NO: 2.

In aspect 5 the present invention includes the variant according to aspect 4, wherein the variant has an amino acid sequence which comprises the amino acid substitutions W70Y and S200N, said positions being defined with reference to SEQ ID NO: 2.

In aspect 6 a variant polypeptide according to the invention has alpha-amylase activity and has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 225 and at least one further substitution of an amino acid residue corresponding to any of amino acids

14, 15, 124, 133, 200, 282, 358,

said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity.

In aspect 7 the present invention includes the variant according to aspect 6, wherein the variant has an amino acid sequence which comprises the amino acid substitution L225F and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2.

In aspect 8 the present invention includes the variant according to aspect 7, wherein the variant has an amino acid sequence which comprises the amino acid substitution L225F and one or more of

I14V, I15V, V124I, S133T, S200N, L282I, L282F and S358A,

said positions being defined with reference to SEQ ID NO: 2.

In aspect 9 the present invention includes the variant according to aspect 6, wherein the variant has an amino acid sequence which comprises a substitution of the amino acid residues corresponding to amino acids 225 and 133 or 200, said positions being defined with reference to SEQ ID NO: 2.

In aspect 10 the present invention includes the variant according to aspect 9, wherein the variant has an amino acid sequence which comprises the amino acid substitutions L225F and S200N, said positions being defined with reference to SEQ ID NO: 2.

In aspect 11 a variant polypeptide according to the invention has alpha-amylase activity and has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 200 and at least one further substitution of an amino acid residue corresponding to any of amino acids

14, 15, 70, 124, 133, 225, 282, 358,

said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity.

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In aspect 12 the present invention includes the variant according to aspect 11, wherein the variant has an amino acid sequence which comprises the amino acid substitution S200N and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2.

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In aspect 13 the present invention includes the variant according to aspect 11, wherein the variant has an amino acid sequence which comprises the amino acid substitution S200N and one or more of I14V, I15V, W70Y, V124I, S133T, S200N, L282I, L282F and S358A, said positions being defined with reference to SEQ ID NO: 2.

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In aspect 14 the present invention includes the variant according to aspect 11, wherein the variant has an amino acid sequence which comprises a substitution of the amino acid residues corresponding to amino acids 133 and 200, said positions being defined with reference to SEQ ID NO: 2.

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In aspect 15 the present invention includes the variant according to aspect 14, wherein the variant has an amino acid sequence which comprises the amino acid substitutions S200N and S133T, said positions being defined with reference to SEQ ID NO: 2.

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In aspect 16 the present invention includes the variant according to any one of aspects 1 to 15, wherein the variant has an amino acid sequence which comprises one of the following sets of substitutions:

W70Y and S200N;

W70Y and L282F;

W70Y and L282I;

W70Y and S133T;
W70Y and L225F;
W70Y and I14V;
W70Y and I15V;
W70Y and S358A;
W70Y and V124I;
W70Y, S200N and L225F;
W70Y, L282F and L225F;
W70Y, L282I and L225F;
W70Y, S133T and L225F;
W70Y, S200N and S358A;
W70Y, L282F and S358A;
W70Y, L282I and S358A;
W70Y, S133T and S358A;
W70Y, S200N and V124I;
W70Y, L282F and V124I;
W70Y, L282I and V124I;
W70Y, S133T and V124I;
W70Y, S200N and S133T;
W70Y, L282F and S133T;
W70Y, L282I and S133T;
W70Y, S200N, S133T and L225F;
W70Y, L282F, S133T and L225F;
W70Y, L282I, S133T and L225F;
W70Y, S200N, S133T and S358A;
W70Y, L282F, S133T and S358A;
W70Y, S200N, S133T and V124I;
W70Y, L282F, S133T and V124I;
L225F and I14V;
L225F and I15V;
L225F and S358A;
L225F and V124I;
L225F, S358A and I14V;
L225F, V124I and I15V;

S200N, L225F and I14V;
 S200N, L225F and I15V;
 S200N, L225F and S358A;
 S200N, L225F and V124I;
 S200N, L225F, S358A and I14V;
 S200N, L225F, V124I and I15V;
 S200N, L282F, S133T and L225F;
 S200N, L282I, S133T and L225F;
 S200N, L282F, S133T, L225F and S358A;
 S200N, L282I, S133T, L225F and S358A;
 S200N, L282F, S133T, L225F and V124I; or
 S200N, L282I, S133T, L225F and V124I,

said positions being defined with reference to SEQ ID NO: 2.

In aspect 17 the present invention includes the variant according to any one of aspects 1 to 16, wherein the one or more altered properties include an increased thermostability.

5 In aspect 18 the present invention includes the variant according to aspect 17, wherein the one or more altered properties include an increased thermostability at pH 4.

In aspect 19 the present invention includes the variant according to aspect 17, wherein the one or more altered properties include an increased thermostability at pH 5.

10 In aspect 20 the present invention includes the variant according to aspect 17, wherein the one or more altered properties include an increased thermostability at pH 7.

In aspect 21 the present invention includes the variant according to any one of aspects 1 to 16, wherein the one or more altered properties include an increased sucrose tolerance.

15 In aspect 22 the present invention includes the variant according to aspect 21, wherein the one or more altered properties include an increased sucrose tolerance at pH 4.

In aspect 23 the present invention includes the variant according to aspect 21, wherein the one or more altered properties include an increased sucrose tolerance at pH 5.

In aspect 24 the present invention includes the variant according to aspect 21, wherein the one or more altered properties include an increased sucrose tolerance at pH 7.

In aspect 25 the present invention includes the variant according to any one of aspects 1 to 16, wherein the one or more altered properties include an increased Activity at pH4 : Activity at pH5 ratio.

5 In aspect 26 the present invention includes the variant according to any one of aspects 1 to 16, wherein the one or more altered properties include an increased Activity at pH7 : Activity at pH5 ratio.

10 In aspect 27 the present invention includes the variant according to any one of aspects 1 to 16, wherein the one or more altered properties include an increased thermostability in the presence of sucrose.

In aspect 28 the present invention includes the variant according to aspect 27, wherein the one or more altered properties include an increased thermostability at pH 4 in the presence of sucrose.

15 In aspect 29 the present invention includes the variant according to aspect 27, wherein the one or more altered properties include an increased thermostability at pH 5 in the presence of sucrose.

In aspect 30 the present invention includes the variant according to aspect 27, wherein the one or more altered properties include an increased thermostability at pH 7 in the presence of sucrose.

20 In aspect 31 the present invention includes the variant according to any one of aspects 1 to 16, wherein the one or more altered properties include an increased specific activity.

In aspect 32 the present invention includes the variant according to aspect 31, wherein the one or more altered properties include an increased specific activity at pH 4.

25 In aspect 33 the present invention includes the variant according to aspect 31, wherein the one or more altered properties include an increased specific activity at pH 5.

In aspect 34 the present invention includes the variant according to aspect 31, wherein the one or more altered properties include an increased specific activity at pH 7.

30 In aspect 35 the present invention includes the variant according to any one of aspects 1 to 16, wherein the altered property includes an increased specific activity and an increased thermostability.

In aspect 36 the present invention includes the variant according to aspect 35, wherein the altered property includes an increased specific activity at pH 4 and an increased thermostability.

5 In aspect 37 the present invention includes the variant according to aspect 35, wherein the altered property includes an increased specific activity at pH 5 and an increased thermostability.

In aspect 38 the present invention includes the variant according to aspect 35, wherein the altered property includes an increased specific activity at pH 7 and an increased thermostability.

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In aspect 39 the present invention includes the variant according to any one of aspects 1 to 16, wherein the altered property includes an increased thermostability compared with the reference polypeptide as set out in SEQ ID NO: 2, while the specific activity at pH 5 of the variant has reduced by at most 20% as compared with the reference polypeptide.

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In aspect 40 the present invention includes the variant according to aspect 39, wherein specific activity at pH 5 of the variant has reduced by at most 30%, in an aspect by at most 40%, in an aspect by at most 50% as compared to the reference polypeptide

20

In aspect 41 the present invention includes the variant according to any one of aspects 1 to 16, wherein the altered property includes an increased thermostability compared with the reference polypeptide as set out in SEQ ID NO: 2, while the specific activity at pH 7 of the variant has reduced by at most 20% as compared with the reference polypeptide.

In aspect 42 the present invention includes the variant according to aspect 41, wherein specific activity at pH 7 of the variant has reduced by at most 30%, in an aspect by at most 40%, in an aspect by at most 50% as compared to the reference polypeptide

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In aspect 43 the present invention includes the variant according to any one of aspects 1 to 42, wherein the reference polypeptide having alpha-amylase activity is the polypeptide as set out in SEQ ID NO: 2.

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In aspect 44 the present invention includes the variant according to any one of aspects 1 to 42, wherein the reference polypeptide having alpha-amylase activity is the polypeptide as set out in SEQ ID NO: 2 which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises the amino acid substitution W70Y, said position being defined with reference to SEQ ID NO: 2.

In aspect 45 the present invention includes the variant according to any one of aspects 1 to 42, wherein the reference polypeptide having alpha-amylase activity is the polypeptide as set out in SEQ ID NO: 2 which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises the amino acid substitution L225F, said position being defined with reference to SEQ ID NO: 2.

In aspect 46 the present invention includes the variant according to any one of aspects 1 to 42, wherein the reference polypeptide having alpha-amylase activity is the polypeptide as set out in SEQ ID NO: 2 which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises the amino acid substitution S200N, said position being defined with reference to SEQ ID NO: 2.

In aspect 47 the present invention includes the variant according to any one of aspects 1 to 5, wherein the variant has an amino acid sequence which comprises a substitution of the amino acid residues corresponding to amino acids 70 and 200, preferably W70Y and S200N, said positions being defined with reference to SEQ ID NO: 2, and wherein the altered property includes one or more of

- a) an increased thermostability;
 - b) an increased sucrose tolerance;
 - c) an increased thermostability in the presence of sucrose
 - d) an increased Activity at pH4 : Activity at pH5 ratio;
 - e) an increased Activity at pH7 : Activity at pH5 ratio; and
 - f) an increased specific activity,
- as compared to a reference polypeptide having alpha-amylase activity as set out in SEQ ID NO: 2.

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In aspect 48 the present invention includes a nucleic acid sequence encoding a variant polypeptide according to any one of the preceding claims.

In aspect 49 the present invention includes a nucleic acid construct comprising the nucleic acid sequence of aspect 48 operably linked to one or more control sequences capable of directing the expression of an alpha-amylase in a suitable expression host.

In aspect 50 the present invention includes a recombinant expression vector comprising the nucleic acid construct of aspect 49.

In aspect 51 the present invention includes a recombinant host cell comprising the expression vector of aspect 50.

In aspect 52 the present invention includes a method for producing an alpha-amylase comprising cultivating the host cell of aspect 51 under conditions conducive to production of the alpha-amylase and recovering the alpha-amylase.

- 5 In aspect 53 the present invention includes a composition comprising the variant polypeptide according to any one of aspects 1 to 47 or obtainable by the method according to aspect 52 and one or more components selected from the group consisting of milk, gluten, granulated fat, an additional enzyme, an amino acid, a salt, oxidants, reducing agents, emulsifiers, gums, flavours, acids, starch, modified starch, humectants
10 and preservatives.

In aspect 54 the present invention includes a pre-mix comprising flour and the variant polypeptide according to any one of aspects 1 to 47 or obtainable by the method according to aspect 52.

- 15 In aspect 55 the pre-mix of aspect 54 includes at least one additional enzyme.

In aspect 56 the present invention includes use of the variant polypeptide according to any one of aspects 1 to 47 or of the composition according to aspect 53 or of the pre-mix according to aspect 54 or 55 in the preparation of a dough and/or a baked product.

- In aspect 57 the present invention includes a dough comprising the variant polypeptide
20 according to any one of aspects 1 to 47 or the composition according to aspect 53 or the pre-mix according to aspect 54 or 55.

In aspect 58 the present invention includes a process for the production of a baked product, which method comprises baking the dough according to aspect 57.

- In aspect 59 the present invention includes a baked product obtainable by the process
25 according to aspect 58 or by the use according to aspect 56.

In aspect 60 the present invention includes a method of producing an alpha-amylase polypeptide variant, which method comprises:

- a) selecting an alpha-amylase polypeptide;
30 b) substituting at least one amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue corresponding to any of amino acids
14, 15, 124, 133, 225, 200, 282, 358,
said positions being defined with reference to SEQ ID NO: 2;

- c) optionally substituting one or more further amino acids as defined in b);
d) preparing the variant resulting from steps a)-c);
e) determining a property of the variant; and
f) selecting a variant having an altered property in comparison to the
5 polypeptide of a), thereby to produce an alpha-amylase polypeptide variant.

In aspect 61 the present invention includes the method aspect 60, wherein the alpha-amylase polypeptide under a) is an alpha-amylase polypeptide having an amino acid sequence having at least 70% identity to the amino acid as set out in SEQ ID NO: 2.

10

In aspect 62 the present invention includes a method of producing an alpha-amylase polypeptide variant, which method comprises:

- a) selecting an alpha-amylase polypeptide;
b) substituting at least one amino acid residue corresponding to amino acid
15 200 and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 70, 124, 133, 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2;
c) optionally substituting one or more further amino acids as defined in b);
20 d) preparing the variant resulting from steps a)-c);
e) determining a property of the variant; and
f) selecting a variant having an altered property in comparison to the polypeptide of a), thereby to produce an alpha-amylase polypeptide variant.

- 25 In aspect 63 the present invention includes the method aspect 62, wherein the alpha-amylase polypeptide under a) is an alpha-amylase polypeptide having an amino acid sequence having at least 70% identity to the amino acid as set out in SEQ ID NO: 2, preferably the alpha-amylase polypeptide under a) is an alpha-amylase polypeptide having an amino acid sequence as set out in SEQ ID NO: 2.

30

In aspect 64 the present invention includes the use of a variant polypeptide according to any one of aspects 1 to 47 or of the composition according to aspect 53 or of the pre-mix according to aspect 54 or 55 in the food industry.

In aspect 65 the present invention includes the variant according to any one of aspects 1 to 16, wherein the one or more altered properties includes an increased ratio of the activity at a temperature of 50 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

In aspect 66 the present invention includes the variant according to aspect 65, wherein the one or more altered properties includes an increased ratio of the activity at a temperature of 60 degrees Celsius or higher versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

In aspect 67 the present invention includes the variant according to aspect 65, wherein the one or more altered properties includes an increased ratio of the activity at a temperature of 60 degrees Celsius versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

In aspect 68 the present invention includes the variant according to aspect 65, wherein the one or more altered properties includes an increased ratio of the activity at a temperature of 80 degrees Celsius versus the activity at a temperature of 37 degrees Celsius as compared to a reference polypeptide having alpha-amylase activity (such as the polypeptide of SEQ ID NO: 2) measured under the same conditions.

In aspect 69 the present invention includes the variant according to any one of aspects 1 to 5, wherein the variant has an amino acid sequence which comprises a substitution of the amino acid residues corresponding to amino acids 70 and 200, preferably W70Y and S200N, said positions being defined with reference to SEQ ID NO: 2, and wherein the altered property includes an increased thermostability as compared to a reference polypeptide having alpha-amylase activity as set out in SEQ ID NO: 2.

In aspect 70 the present invention includes the use of a variant polypeptide according to any one of aspects 65 to 69 in the food industry.

In aspect 71 the present invention includes a composition comprising the variant polypeptide according to any one of aspects 65 to 69 and one or more components selected from the group consisting of milk, gluten, granulated fat, an additional enzyme, an amino acid, a salt, oxidants, reducing agents, emulsifiers, gums, flavours, acids, starch, modified starch, humectants and preservatives.

In aspect 72 the present invention includes a pre-mix comprising flour and the variant polypeptide according to any one of aspects 65 to 69.

In aspect 73 the present invention includes a dough comprising the variant polypeptide according to any one of aspects 65 to 69.

5 In aspect 74 the present invention includes a process for the production of a baked product, which method comprises baking the dough according to aspect 73.

10 The above-mentioned industrial applications of the alpha-amylase enzyme according to the invention comprise only a few examples and this listing is not meant to be restrictive.

Other uses of the alpha-amylase variant according to the invention may include:

- the production of glucose, fructose and maltose syrups;
- production of starch hydrolysates such as maltodextrins;
- production of modified starches;
- 15 - modification of starch components in animal feed;
- replacement of malt in brewing;
- use in a glue including wall paper paste;
- use in plastic objects made using starch, including plastic bags made from polymerized starch films; and/or
- 20 - use in waste bread reprocessing.

25 A reference herein to a patent document or other matter which is given as prior art is not to be taken as an admission that that document or matter was known or that the information it contains was part of the common general knowledge as at the priority date of any of the claims.

The disclosure of each reference set forth herein is incorporated herein by reference in its entirety.

The present invention is further illustrated by the following non-limiting examples:

30

Examples

A reference polypeptide also referred to as a parent polypeptide as set out in SEQ ID NO: 2 may be produced as described in US Patent US 8,426,182 B1.

Materials & Methods

NBAU Assay

Enzymatic activity of the alpha-amylase variant and of the parent polypeptide may
5 be expressed as NBAU. One NBAU is defined as the amount of enzyme resulting in
the release of 1 μ mole of pNP (*para*-nitrophenol) per minute using the end blocked
pNP-G7 Ceralpha substrate at pH = 5.2 and T = 37°C.

10 The principle of the NBAU activity test originates from a (manual) Megazyme α -
amylase kit test (Ceralpha). The assay was made suitable for analyzer application.
The assay is executed at pH 5.20 taking into account the pH optima for α -
glucosidase and amyloglucosidase (pH range 5 - 6). The test is performed with a
Konelab Arena 30 analyzer (Thermo Scientific, Vantaa, Finland).

15 The enzymatic activity is determined at 37°C and pH 5.20 using a non-reducing-end
blocked p-nitrophenyl maltoheptaoside substrate (= BPNPG7, Ceralpha) combined
with excess levels of thermostable α -glucosidase and amyloglucosidase (both from
Ceralpha: α -Amylase Reagent R-CAAR4, Megazyme, Ireland). Hydrolysis of the
BPNPG7 substrate by an alpha-amylase results in p-nitrophenyl maltosaccharide
20 fragments. The reaction is terminated (and colour developed) by the addition of an
alkaline solution. The absorbance at a wavelength of 405 nm is determined and is a
measure for enzymatic activity. Activity is calculated from a molar extinction
coefficient determination, through a calibration with a para-nitrophenol solution of
known concentration.

25

1) AACC Method 22-02.01

Measurement of alpha-amylase in Plant and Microbial Materials Using the Ceralpha® Method

30 The alpha-amylase activity is analysed by measuring activity using a Megazyme
CERALPHA alpha-amylase assay kit (Megazyme International Ireland Ltd., Co. Wicklow,
Ireland) according to the manufacturer's instruction. All variants described in the examples
showed alpha-amylase activity in the CERALPHA assay. The reference polypeptide as set
out in SEQ ID NO: 2 showed alpha-amylase activity in the CERALPHA assay.

2)**Assay 2A Maltotriose Assay also referred to as Activity at pH 5**

This assay may be used to determine Activity on maltotriose substrate.

One Maltotriose Unit (MU) is defined as the amount of enzyme that liberates 1 μ mole glucose per minute using maltotriose substrate under the following assay conditions. Enzymatic activity is determined in a 30 minutes incubation at 37°C and pH 5.0 using maltotriose as substrate. Enzymatic hydrolysis of maltotriose results in quantitative release of glucose, which is a measure for enzymatic activity.

Samples of approximately 0.4- 4 mg/ml protein are diluted to a range between 0.0125 and 0.125 MU/ml in 100 mM citric acid buffer containing 1 g/L BSA, that has been adjusted to pH 5.0 using 4 N NaOH. 10 mg/ml maltotriose substrate is prepared in 2.5 mM NaCl in MQ water. 160 microliter substrate is preheated for approximately 30 minutes in a PCR thermocycler set at 37°C in a 96 wells PCR plate. 40 microliter of diluted sample is added to the preheated substrate in the thermocycler and mixed well by pipetting up and down several times. 30 minutes after sample addition, 20 microliter of 0.33 N NaOH is added and mixed well to terminate the reaction, and the PCR plate is taken out of the thermocycler. Released glucose is measured by incubation of 55 microliter of the terminated reaction mixture with 195 microliter of hexokinase monoreagent (Ecoline Glucose Hexokinase FS, DiaSys Diagnostic systems GmbH, Holzheim, Germany) for 15 minutes at room temperature in a flat bottom 96 wells plate. Air bubbles are removed from the surface by centrifugation, after which the absorbance at 340 nm is read using a microtiter plate reader. The amount of glucose released is determined relative to a glucose calibration line.

Assay 2B Sucrose tolerance at pH 5

As under Assay 2A), except that the substrate consists of 10 mg/ml maltotriose to which 6.25 mg/ml sucrose is added.

Assay 2C Thermostability at pH 5

Samples of approximately 0.4- 4 mg/ml protein are diluted 75 fold in 100 mM citric acid buffer containing 1 g/L BSA, that has been adjusted to pH 5.0 using 4 N NaOH. 100 microliter of these diluted samples is transferred to a 96 wells PCR plate and is exposed to a 30 minutes incubation at 79.3°C, followed by immediate cooling to 4°C in a PCR thermocycler. Temperature treated samples are then further diluted 100 fold in 100 mM

citric acid buffer containing 1 g/L BSA, that has been adjusted to pH 5.0 using 4 N NaOH. Residual activity is determined as under Assay 2A.

Assay 2D Activity at pH4

- 5 As under Assay 2A, except that the samples are diluted in 100 mM citric acid buffer containing 1 g/L BSA, that has been adjusted to pH 4.0 using 4 N NaOH.

Assay 2E Thermostability at pH 4

- 10 Samples of approximately 0.4- 4 mg/ml protein are diluted 75 fold in 100 mM citric acid buffer citric acid buffer containing 1 g/L BSA, that has been adjusted to pH 4.0 using 4 N NaOH. 100 microliter of these diluted samples is transferred to a 96 wells PCR plate and is exposed to a 30 minutes incubation at 57.8°C, followed by immediate cooling to 4°C in a PCR thermocycler. Temperature treated samples are then further diluted 100 fold in 100 mM citric acid buffer containing 1 g/L BSA, that has been adjusted to pH 5.0 using 4 N NaOH. Residual activity is determined as under Assay 2A.

Assay 2F Thermostability at pH 5 in the presence of sucrose

- 20 Samples of approximately 0.4- 4 mg/ml protein are diluted 75 fold in 100 mM citric acid buffer containing 1 g/L BSA, that has been adjusted to pH 5.0 using 4 N NaOH, with 2.5% sucrose added. 100 microliter of these diluted samples is transferred to a 96 wells PCR plate and is exposed to a 30 minutes incubation at 79.3°C, followed by immediate cooling to 4°C in a PCR thermocycler. Temperature treated samples are then further diluted 100 fold in 100 mM citric acid buffer containing 1 g/L BSA, that has been adjusted to pH 5.0 using 4 N NaOH. Residual activity is determined as under A).

Assay 2G Activity at pH7

- 25 As under Assay 2A, except that the samples are diluted to approximately 0.03 – 0.3 MU/ml in 50 mM sodium phosphate buffer containing 1 g/L BSA, that has been adjusted to pH 7.0 using 4 N NaOH.

Assay 2H Sucrose tolerance at pH 7

- 30 As under Assay 2G, except that the substrate consisted of 10 mg/ml maltotriose to which 18.75 mg/ml sucrose is added.

Assay 2J Thermostability at pH 7

Samples of approximately 0.4- 4 mg/ml protein are diluted 50 fold in 50 mM sodium phosphate buffer containing 1 g/L BSA, that has been adjusted to pH 7.0 using 4 N NaOH. 100 microliter of these diluted samples is transferred to a 96 wells PCR plate and is exposed to a 10 minutes incubation at 76.5 °C, followed by immediate cooling to 4 °C in a PCR thermocycler. Temperature treated samples are then further diluted 80 fold in 100 mM citric acid buffer containing 1 g/L BSA, that has been adjusted to pH 5.0 using 4 N NaOH. Residual activity is determined as under Assay 2A.

Assay 2K Thermostability at pH 7 in the presence of sucrose

Samples of approximately 0.4- 4 mg/ml protein are diluted 50 fold in 50 mM sodium phosphate buffer containing 1 g/L BSA, that has been adjusted to pH 7.0 using 4 N NaOH, with 5% sucrose added. 100 microliter of these diluted samples is transferred to a 96 wells PCR plate and is exposed to a 10 minutes incubation at 76.5 °C, followed by immediate cooling to 4 °C in a PCR thermocycler. Temperature treated samples are then further diluted 80 fold in 100 mM citric acid buffer containing 1 g/L BSA, that has been adjusted to pH 5.0 using 4 N NaOH. Residual activity is determined as under 2A).

Assay 2L Protein content

Protein content is determined using the Bradford assay (Bradford, M.M. (1976), "Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", *Anal. Biochem.* **72**: 248–254) in combination with a SDS-PAGE verification to confirm that samples show >90% purity based on band intensity.

Assay 2M Sucrose tolerance at pH 4

As under Assay 2B, except that samples of approximately 0.4- 4 mg/ml protein are diluted 75 fold in 100 mM citric acid buffer citric acid buffer containing 1 g/L BSA, that has been adjusted to pH 4.0 using 4 N NaOH.

Assay 2N Thermostability at pH 4 in the presence of sucrose

As under Assay 2K, except that samples of approximately 0.4- 4 mg/ml protein are diluted 50 fold in 100 mM citric acid buffer citric acid buffer containing 1 g/L BSA, that has been adjusted to pH 4.0 using 4 N NaOH, with 2.5 - 5% sucrose added.

Assay 2P at Activity at pH 5 60°C

As under Assay 2A), except the PCR thermocycler is set at 60°C, in which substrate is preheated for approximately 15 minutes. The reaction is terminated 10 minutes after sample addition.

Assay 2Q Activity at pH 5 at 80°C

As under Assay 2A), except the PCR thermocycler is set at 80°C, in which substrate is preheated for approximately 15 minutes. The reaction is terminated 10 minutes after sample addition.

Determination of altered properties.

Altered properties of alpha-amylase variants according to the invention as compared with a reference polypeptide, preferably having an amino acid sequence as set out in SEQ ID NO: 2, include one or more of

- a) an increased thermostability;
- b) an increased sucrose tolerance;
- c) an increased thermostability in the presence of sucrose;

To determine altered properties a) to c) at pH 4, 5 or 7 the following steps are followed.

Firstly, the properties of the variants and the reference polypeptide are measured as described under Assay 2A to Assay 2K and Assays 2M and 2N above.

Secondly, from these measurements it is determined if the property is altered as compared to the reference polypeptide. Examples of such determination and calculation is described below for thermostability and sucrose tolerance. For thermostability in the presence of sucrose this can be done analogously to the description for thermostability.

Preferably the values obtained in the assay are an average of at least 2 measurements on the same sample.

Exemplary determination and calculation of sucrose tolerance of variant #

The activity of variant # is determined in the presence of sucrose (measured as described under Assay 2B) and expressed as a ratio to the activity measured for the same variant in the absence of sucrose (measured as described under Assay 2A above). The reference polypeptide (the alpha-amylase having an amino acid sequence as set out in SEQ ID NO: 2) is subjected to the same experimental conditions.

If the activity of variant # would be 850 units/ml in presence of sucrose (Assay 2B), and 1000 units/ml in absence of sucrose (Assay 2A), the ratio of the activity in presence of sucrose to the activity in absence of sucrose for variant # would be 0.85.

5 If the activity of the reference polypeptide would be 600 units/ml in presence of sucrose (Assay 2B), and 1000 units/ml in absence of sucrose (Assay 2A), then the ratio of the activity in presence of sucrose to the activity in absence of sucrose for the reference polypeptide would be 0.60.

This value of the reference polypeptide is then normalized to 100%.

10 In this exemplary calculation the sucrose tolerance of variant # compared with the reference polypeptide would then be $(0.85/0.60) \times 100\% = 142\%$. 142% (for variant #) is an increase compared with 100% (for the reference polypeptide), as a result variant # is said to have an increased sucrose tolerance compared with the reference polypeptide.

Exemplary determination and calculation of thermostability of variant

15 The thermostability activity of variant # is determined at a certain pH e.g. at pH 5.

The thermostability at pH 5 may be expressed as the ratio of

[Residual Activity of a variant determined after an incubation at a temperature of above 37 degrees Celsius at pH 5] to [Activity of the variant determined after an incubation at a temperature of 37 degrees Celsius at pH 5],

20 expressed as a percentage of the ratio of

[Residual Activity of the reference polypeptide after an incubation at a temperature of above 37 degrees Celsius at pH 5] to [Activity of the reference polypeptide after an incubation at a temperature of 37 degrees Celsius at pH 5].

25 The Residual activity of variant # after an incubation at a temperature of above 37 degrees Celsius at pH 5 is determined (measured as described under Assay 2C) and expressed as the ratio to the Activity of the variant determined after an incubation at a temperature of 37 degrees Celsius at pH 5 (measured as described under Assay 2A above).

30 The reference polypeptide (the alpha-amylase having an amino acid sequence as set out in SEQ ID NO: 2) is subjected to the same experimental conditions.

If the Residual activity of variant # would be 350 units/ml after incubation at elevated temperature (Assay 2C), and the Activity after incubation at a temperature of 37 degrees Celsius would be 1000 units/ml (Assay 2A), the ratio of the residual activity after

incubation at elevated temperature to the activity after incubation at a temperature of 37 degrees Celsius for variant # would be 0.35.

If the Residual activity of the reference polypeptide in Assay 2C would be 200 units/, and the Activity in Assay 2A would be 1000 units/ml, the ratio of the Residual activity after incubation at elevated temperature to the Activity after incubation at a temperature of 37 degrees Celsius for the reference polypeptide would be 0.20. This value of the reference polypeptide is then normalized to 100%.

In this exemplary calculation the thermostability at pH5 of variant # compared with the reference polypeptide would then be $(0.35/0.20) \times 100\% = 175\%$. 175% (for variant #) is an increase compared with 100% (for the reference polypeptide), as a result variant # is said to have an increased thermostability, in particular increased thermostability at pH5 compared with the reference polypeptide.

Strains and plasmids

Bacillus subtilis strain BS154 (CBS 363.94) ($\Delta aprE$, $\Delta nprE$, $amyE^-$, spo^-) is described in Quax and Broekhuizen 1994 Appl Microbiol Biotechnol. 41: 425-431.

The *E. coli* / *B. subtilis* shuttle vector pBHA12 is described in (WO2008/000632).

Alicyclobacillus pohliae NCIMB14276 is described by Imperio et al (Int. J. Syst. Evol. Microbiol 58:221-225, 2008).

Molecular biology techniques

Molecular biology techniques known to the skilled person are performed according to (Sambrook & Russell, Molecular Cloning: A Laboratory Manual, 3rd Ed., CSHL Press, Cold Spring Harbor, NY, 2001). Polymerase chain reaction (PCR) is performed on a thermocycler with Phusion High-Fidelity DNA polymerase (Finnzymes OY, Espoo, Finland) according to the instructions of the manufacturer.

Example 1

DNA constructs and transformation

The multipurpose integration expression vector pDBC1 is designed to make use of type two S restriction enzyme cloning to combine expression modules and genes of interest. An example of a type two S restriction enzyme is *BsmBI*. The *amyE* integration vector pBest4 as described in WO2008/148575 is modified by replacing the spectinomycin marker and *lacZ* gene with a synthetic DNA fragment (SEQ ID NO: 3).

Vector pBest4 is digested with *Tth*III and *Asi*I and the synthetic DNA fragment (SEQ ID NO: 3) containing a *Tth*III site, 340 bp of 5'-*amyE*, *Bsm*BI site, chloramphenicol selection marker, *amyM* terminator, *Bsm*BI site, lox 66 site, spectinomycin selection marker, lox71 site, 120 bp of 3'-*amyE* and *Asi*I site is inserted which resulted in vector pDBC1 (Fig. 1).

The G01 expression module (SEQ ID NO: 4) contains the P15 promoter, a modified RNA leader sequence as described in EP2186880 (nucleotides 31-251 of SEQ ID NO: 70 therein) a *Nde*I site at the ATG start and two *Bsm*BI sites at the 5' and 3' ends. The expression module G01 is made synthetically.

Amino acid changes that are introduced in the alpha-amylase variants are depicted in Table 3. Positions of the amino acid change are indicated in comparison with SEQ ID NO: 2 (an example of a reference polypeptide having alpha-amylase activity).

Table 3: Amino acid changes to be introduced in the parent polypeptide, wherein the parent polypeptide has an amino acid sequence as set out in SEQ ID NO 2: Amino acids are depicted according to the single letter annotation

Variant#	Amino acid change or changes *)
V01	W70Y and S200N
V02	W70Y and L282F
V03	W70Y and L282I
V04	W70Y and S133T
V05	W70Y and L225F
V06	W70Y and I14V
V07	W70Y and I15V
V08	W70Y and S358A
V09	W70Y and V124I
V10	W70Y, S200N and L225F
V11	W70Y, L282F and L225F
V12	W70Y, L282I and L225F
V13	W70Y, S133T and L225F
V14	W70Y, S200N and S358A
V15	W70Y, L282F and S358A
V16	W70Y, L282I and S358A

V17	W70Y, S133T and S358A
V18	W70Y, S200N and V124I
V19	W70Y, L282F and V124I
V20	W70Y, L282I and V124I
V21	W70Y, S133T and V124I
V22	W70Y, S200N and S133T
V23	W70Y, L282F and S133T
V24	W70Y, L282I and S133T
V25	W70Y, S200N, S133T and L225F
V26	W70Y, L282F, S133T and L225F
V27	W70Y, L282I, S133T and L225F
V28	W70Y, S200N, S133T and S358A
V29	W70Y, L282F, S133T and S358A
V30	W70Y, S200N, S133T and V124I
V31	W70Y, L282F, S133T and V124I
V32	L225F and I14V
V33	L225F and I15V
V34	L225F and S358A
V35	L225F and V124I
V36	L225F, S358A and I14V
V37	L225F, V124I and I15V
V38	S200N, L225F and I14V
V39	S200N, L225F and I15V
V40	S200N, L225F and S358A
V41	S200N, L225F and V124I
V42	S200N, L225F, S358A and I14V
V43	S200N, L225F, V124I and I15V
V44	S200N, L282F, S133T and L225F
V45	S200N, L282I, S133T and L225F
V46	S200N, L282F, S133T, L225F and S358A
V47	S200N, L282I, S133T, L225F and S358A
V48	S200N, L282F, S133T, L225F and V124I
V49	S200N, L282I, S133T, L225F and V124I

V50	Reference alpha-amylase having an amino acid sequence as set out in SEQ ID NO 2
V51	S200N
V52	W70Y
V53	L282F
V54	L282I
V55	S133T
V56	L225F
V57	I14V
V58	I15V
V59	S358A
V60	V124I

*) W70Y and S200N means 2 changes: the amino acid at position 70 and the amino acid at position 200 are changed. The W (Tryptophan) at position 70 is changes into a Y (Tyrosine) and that S (Serine) at position 200 is changed into a N (Asparagine), etc.

- 5 SEQ ID NO: 2 sets out the amino acid sequence of the mature *Alicyclobacillus pohliae* NCIMB14276 wild type alpha-amylase polypeptide without the first 33 amino acids encoding the signal peptide.

SSSASVKGDVIYQIIIDRFYDGDTTNNNPAKSYGLYDPTKSKWKMYWGGDLEGVRQKL
 PYLKQLGVTTIWLSPVLDNLDTLAGTDNTGYHGYWTRDFKQIEEHFGNWTTFDTLVND
 10 AHQNGIKVIVDFVPNHSTPFKANDSTFAEGGALYDNGTYMGNYFDDATKGYFHHNGDI
 SNWDDRYEAQWKNFTDPAGFSLADLSQENGITIAQYLTDAAVQLVAHGADGLRIDAVKH
 FNSGFSKSLADKLYQKKDIFLVGEWYGDDPGAANHLEKVRYANNSGVNVLDFDLNTVI
 RNVFGTFTQTMYDLNNMVNQTGNEYKYKENLITFIDNHDMRSRLTVNSNKANLHQALA
 FILTSRGTPSIYYGTEQYMAGGNDPYNRGMMPAFDTTTTAFKEVSTLAGLRRNNAAIQY
 15 GTTTQRWINNDVYIYERKFFNDVVLVAINRNTQSSYSISGLQTALPNGNYADYLSGLLG
 GNGISVSNGSVASFTLAPGAVSVWQYSTSASAPQIGSVAPNMGIPGNVVTIDGKGFGTT
 QGTVTFGGVTATVKSWTSNRIEVYVPNMAAGLTDVKVTAGGVSSNLYSYNILSGTQTS
 VVFTVKSAPPTNLGDKIYLTGNIPELGNWSTDTS GAVNNAQGPELLAPNYPDWFYVFSVP
 AGKTIQFKFFIKRADGTIQWENGSNHVATTPTGATGNITVTWQN (SEQ ID NO:2).

20

Synthetic DNA constructs containing the nucleic acid sequence encoding the alpha-amylase variants also contain a *BsmBI* restriction site at the 5' end, as shown in SEQ ID

NO: 5 and a double stop codon and *BsmBI* restriction site at the 3' end as shown in SEQ ID NO: 6.

5 The two *BsmBI* sites are added to the 5' ends of both nucleic acids to allow cloning of the synthetic DNA fragments into pDBC1.

The StarGate type two S restriction enzyme cloning system (IBA, GmbH, Göttingen, Germany) is used according to the instructions of the manufacturer to assemble the integration vectors. As an example, a synthetic DNA construct existing of a *BsmBI*, wild type *DSM-AM* sequence as set out in SEQ ID NO: 1, double stop codon and *BsmBI* restriction sites is listed as SEQ ID NO: 7.

10 SEQ ID NO 7: sets out the polynucleotide sequence of a synthetic DNA construct exciting of a *BsmBI* site, wild type *DSM-AM* sequence as set out in SEQ ID NO: 1, double stop codon and *BsmBI* restriction site .

15 All nucleic acid sequence encoding the alpha-amylase variants are designed in a similar fashion and cloned as *BsmBI*, fragments in vector pDBC1 together with the expression module G01. For instance the pDBC1 vector containing the wild type *DSM-AM* sequence as set out in SEQ ID NO: 1 and the G01 expression module is named pDBC-AM1 (Fig. 2).

20 These vectors are transformed to *B. subtilis* strain BS154. The transformants are selected on spectinomycin (100 µg/ml) containing agar plates. The *amyE* regions on pDBC1 targeted the constructs to the *amyE* locus. The double cross over transformants are spectinomycin resistant whereas the undesired single cross-over transformants are also erythromycin resistant. The genomic insertions are confirmed by DNA sequencing. The *B. subtilis* BS154 strains containing the DSM-AM insert and the strain producing the reference polypeptide is named DSM-AM1 and the strains expressing the *DSM-AM* variants are named DSM-AMV01 until DSM-AMV60.

Example 2

30 Expression of alpha-amylase variants, also referred to as *DSM-AM* variants in shake flasks

The *Bacillus subtilis* strains harboring the *DSM-AM* gene variants are placed on 2*TY agar plates and grown for 24 hours at 37°C. A pre-culture of 20 ml 2*TY medium composed of 1.6% (w/v) Bacto tryptone, 1% (w/v) Yeast extract and 0.5% (w/v) NaCl in 100 ml Erlenmeyer flasks are inoculated with the *B. subtilis* cells taken from the plates.

The cultures are shaken vigorously at 37°C and 250 rpm for 16 hours and 0.2 ml culture medium is used to inoculate 20 ml SMM medium. SMM pre-medium contains 1.25% (w/w) yeast extract, 0.05% (w/w) CaCl₂, 0.075% (w/w) MgCl₂·6H₂O, 15 µg/l MnSO₄·4H₂O, 10 µg/l CoCl₂·6H₂O, 0.05% (w/w) citric acid, 0.025% (w/w) antifoam 86/013 (Basildon Chemicals, Abingdon, UK). To complete SMM medium, 20 ml of 5% (w/v) maltose and 20 ml of a 200 mM Na-phosphate buffer stock solution (pH 6.8), both prepared and sterilized separately, are added to 60 ml SMM pre-medium. These cultures are incubated for 48 hours at 37°C and 250 rpm. The supernatants are harvested and analyzed for enzyme productivity. The alpha-amylase activity of the *alpha-amylase* variants is measured according to the NBAU Assay as described in above.

Example 3 Preparation of variants and the reference polypeptide.

Variants and the reference polypeptide (number V50) as listed in table 4 below were prepared as follows.

DNA constructs and transformation

DNA constructs were prepared and transformation was performed as described in example 1. The *B. subtilis* BS154 strains containing the DSM-AM insert and the strain producing the reference polypeptide was named DSM-AMV50 and the strains expressing the *DSM-AM* variants were named DSM-AMV01 until DSM-AMV60.

The reference polypeptide named DSM-AMV50 was expressed as described below for the alpha-amylase variants.

Expression of alpha-amylase variants, also referred to as *DSM-AM* variants

The *Bacillus subtilis* strains harboring the *DSM-AM* gene variants are placed on 2*TY agar plates and grown for 24 hours at 37°C. Pre-cultures were grown in 24 deep well plates (Axygen, Union City, USA). A 1 ml pre-culture was made in 2xTY medium composed of 1.6% (w/w) Bacto tryptone, 1% (w/w) yeast extract and 0.5% (w/w) NaCl. The 24 deep well plates were covered by a Breathseal (Greiner bio-one, Frickenhausen, Germany). After overnight growth at 37°C, 550 rpm and 80% humidity in a Microton incubator shaker (Infors AG, Bottmingen, Switzerland), 2 ml of SMM medium in 24 deep well plates (Axygen, Union City, USA) was inoculated with 1% (v/v) of the pre culture. SMM pre-medium contains 1.25% (w/w) yeast extract, 0.05% (w/w) CaCl₂, 0.075% (w/w)

MgCl₂.6H₂O, 15 µg/l MnSO₄.4H₂O, 10 µg/l CoCl₂.6H₂O, 0.05% (w/w) citric acid, 0.025% (w/w) antifoam 86/013 (Basildon Chemicals, Abingdon, UK). To complete SMM medium, 20 ml of 5% (w/v) maltose and 20 ml of a 200 mM Na-phosphate buffer stock solution (pH 6.8), both prepared and sterilized separately, were added to 60 ml SMM pre-medium.

5 These cultures were incubated in a Microton incubator shaker (Infors AG, Bottmingen, Switzerland) for 48 hours at 37°C, 550 rpm and 80% humidity. The supernatants are harvested and analyzed for enzyme productivity. The alpha-amylase activity of the *alpha-amylase* variants is measured according to the NBAU Assay as described in above.

10

All variants showed alpha-amylase activity (as was measured according to the NBAU Assay as described in above)

Table 4. Variants having substitutions as defined with reference to SEQ ID NO: 2.

15 Amino acids are depicted according to the single letter annotation. Reference alpha-amylase having an amino acid sequence as set out in SEQ ID NO 2 is numbered V50.

Variant#	Substitutions *)
V01	W70Y and S200N
V02	W70Y and L282F
V03	W70Y and L282I
V05	W70Y and L225F
V06	W70Y and I14V
V07	W70Y and I15V
V08	W70Y and S358A
V09	W70Y and V124I
V10	W70Y, S200N and L225F
V11	W70Y, L282F and L225F
V12	W70Y, L282I and L225F
V13	W70Y, S133T and L225F
V14	W70Y, S200N and S358A
V15	W70Y, L282F and S358A
V16	W70Y, L282I and S358A
V17	W70Y, S133T and S358A

V18	W70Y, S200N and V124I
V19	W70Y, L282F and V124I
V20	W70Y, L282I and V124I
V21	W70Y, S133T and V124I
V22	W70Y, S200N and S133T
V23	W70Y, L282F and S133T
V24	W70Y, L282I and S133T
V25	W70Y, S200N, S133T and L225F
V26	W70Y, L282F, S133T and L225F
V27	W70Y, L282I, S133T and L225F
V28	W70Y, S200N, S133T and S358A
V29	W70Y, L282F, S133T and S358A
V30	W70Y, S200N, S133T and V124I
V32	L225F and I14V
V33	L225F and I15V
V34	L225F and S358A
V35	L225F and V124I
V36	L225F, S358A and I14V
V37	L225F, V124I and I15V
V38	S200N, L225F and I14V
V39	S200N, L225F and I15V
V40	S200N, L225F and S358A
V41	S200N, L225F and V124I
V42	S200N, L225F, S358A and I14V
V43	S200N, L225F, V124I and I15V
V45	S200N, L282I, S133T and L225F
V46	S200N, L282F, S133T, L225F and S358A
V50	Reference alpha-amylase having an amino acid sequence as set out in SEQ ID NO 2
V51	S200N
V52	W70Y
V53	L282F
V55	S133T

V56	L225F
V57	I14V
V59	S358A
V60	V124I

*) W70Y and S200N means 2 changes: the amino acid at position 70 and the amino acid at position 200 are changed. The W (Tryptophan) at position 70 is changes into a Y (Tyrosine) and that S (Serine) at position 200 is changed into a N (Asparagine), etc.

- 5 Examples 4 to 12 below were performed using variants and the reference polypeptide as set out in SEQ ID NO: 2, all of which were prepared as described in example 3.

Example 4 Sucrose tolerance at pH5 of variants according to the invention

10 Sucrose tolerance of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2) and of variants according to the invention was determined at pH 5 using assay 2A and assay 2B as described herein.

Sucrose tolerance of variants according to the invention having an amino acid sequence according to SEQ ID NO: 2 including the substitutions as indicated in table 5 below and of the reference alpha amylase polypeptide having an amino acid sequence as set out in
15 SEQ ID NO: 2 was determined as described herein under Materials and Methods above (Assay 2A, Assay 2B and "Exemplary determination and calculation of sucrose tolerance of variant #").

The sucrose tolerance at pH 5 of variants according to the invention was determined as the ratio of
20 [Activity of the variant in Assay 2B (as described herein)] to [Activity of the variant in Assay 2A (as described herein)],
expressed as a percentage of the ratio of
[Activity of the reference polypeptide in Assay 2B (as described herein)] to [Activity of the reference polypeptide in Assay 2A (as described herein)].
25 The percentage thus obtained is the Sucrose tolerance at pH 5 as listed in Table 5 below.

A sucrose tolerance at pH 5 of more than 100% shows that the variant has an increased sucrose tolerance compared to the reference polypeptide.

Table 5 Sucrose tolerance at pH 5 of variants according to the invention compared to the reference polypeptide. The sucrose tolerance of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2), was set at 100%. A sucrose tolerance of more than 100% shows that the variant has an increased sucrose tolerance compared to the reference polypeptide.

Variant#	Substitutions *)	Sucrose tolerance at pH 5 of variant compared with sucrose tolerance of reference polypeptide (set at 100%) (average of 2 measurements)
V01	W70Y and S200N	120%
V02	W70Y and L282F	126%
V03	W70Y and L282I	111%
V05	W70Y and L225F	147%
V06	W70Y and I14V	112%
V07	W70Y and I15V	124%
V08	W70Y and S358A	126%
V09	W70Y and V124I	114%
V10	W70Y, S200N and L225F	146%
V11	W70Y, L282F and L225F	166%
V12	W70Y, L282I and L225F	141%
V13	W70Y, S133T and L225F	147%
V14	W70Y, S200N and S358A	129%
V15	W70Y, L282F and S358A	118%
V17	W70Y, S133T and S358A	121%
V18	W70Y, S200N and V124I	113%
V19	W70Y, L282F and V124I	127%
V22	W70Y, S200N and S133T	114%
V23	W70Y, L282F and S133T	123%
V25	W70Y, S200N, S133T and L225F	143%
V26	W70Y, L282F, S133T and L225F	159%
V27	W70Y, L282I, S133T and L225F	137%
V28	W70Y, S200N, S133T and S358A	124%

V29	W70Y, L282F, S133T and S358A	123%
V30	W70Y, S200N, S133T and V124I	114%
V32	L225F and I14V	140%
V33	L225F and I15V	148%
V34	L225F and S358A	155%
V35	L225F and V124I	140%
V36	L225F, S358A and I14V	151%
V37	L225F, V124I and I15V	145%
V38	S200N, L225F and I14V	142%
V39	S200N, L225F and I15V	151%
V40	S200N, L225F and S358A	142%
V41	S200N, L225F and V124I	138%
V42	S200N, L225F, S358A and I14V	157%
V43	S200N, L225F, V124I and I15V	145%
V45	S200N, L282I, S133T and L225F	121%
V46	S200N, L282F, S133T, L225F and S358A	181%

*) W70Y and S200N means 2 changes: the amino acid at position 70 and the amino acid at position 200 are substituted. The W (Tryptophan) at position 70 is changed into a Y (Tyrosine) and that S (Serine) at position 200 is changed into a N (Asparagine), etc.

5 Example 5 Thermostability at pH 5

Thermostability at pH 5 of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2) and of variants according to the invention was determined using assays A2 and 2C as described herein.

Thermostability at pH 5 of variants according to the invention having an amino acid sequence according to SEQ ID NO: 2 including an amino acid change as indicated in table 6 below and of the reference polypeptide having an amino acid sequence as set out in SEQ ID NO: 2 was determined as described herein under Materials and Methods
5 above. This done using Assay 2A, Assay 2C and “Exemplary determination and calculation of thermostability of variant #”

The Thermostability at pH 5 of a variant according to the invention was determined as the ratio of
10 [Activity of the variant in Assay 2C (as described herein)] to [Activity of the variant in Assay 2A (as described herein)],
expressed as a percentage of the ratio of
[Activity of the reference polypeptide in Assay 2C (as described herein)] to [Activity of the reference polypeptide in Assay 2A (as described herein)].
15 The percentage thus obtained is the Thermostability at pH 5 as listed in table 6.

A Thermostability at pH 5 of more than 100% shows that the variant has an increased Thermostability at pH 5 compared to the reference polypeptide.

Table 6 Thermostability at pH 5 of variants according to the invention compared to the
20 reference polypeptide. The Thermostability at pH 5 of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2), was set at 100%. A Thermostability at pH 5 of more than 100% shows that the variant has an increased Thermostability at pH 5 compared to the reference polypeptide .

Variant#	Substitutions *)	Thermostability at pH 5 of variant compared with reference polypeptide (set at 100%) (average of 2 measurements)
V01	W70Y and S200N	248%
V02	W70Y and L282F	188%
V03	W70Y and L282I	185%
V06	W70Y and I14V	163%
V07	W70Y and I15V	166%
V08	W70Y and S358A	176%
V09	W70Y and V124I	172%
V10	W70Y, S200N and L225F	202%
V11	W70Y, L282F and L225F	177%
V12	W70Y, L282I and L225F	145%
V13	W70Y, S133T and L225F	164%
V14	W70Y, S200N and S358A	247%
V15	W70Y, L282F and S358A	181%
V16	W70Y, L282I and S358A	161%
V17	W70Y, S133T and S358A	180%
V18	W70Y, S200N and V124I	220%
V19	W70Y, L282F and V124I	212%
V20	W70Y, L282I and V124I	195%
V21	W70Y, S133T and V124I	252%
V22	W70Y, S200N and S133T	239%
V23	W70Y, L282F and S133T	211%
V25	W70Y, S200N, S133T and L225F	223%
V26	W70Y, L282F, S133T and L225F	199%
V27	W70Y, L282I, S133T and L225F	166%
V28	W70Y, S200N, S133T and S358A	253%
V29	W70Y, L282F, S133T and S358A	206%
V30	W70Y, S200N, S133T and V124I	245%
V38	S200N, L225F and I14V	139%

V41	S200N, L225F and V124I	139%
V42	S200N, L225F, S358A and I14V	142%
V45	S200N, L282I, S133T and L225F	172%
V46	S200N, L282F, S133T, L225F and S358A	218%

*) W70Y and S200N means 2 changes: the amino acid at position 70 and the amino acid at position 200 are substituted. The W (Tryptophan) at position 70 is changed into a Y (Tyrosine) and that S (Serine) at position 200 is changed into a N (Asparagine), etc.

5 **Example 6 Activity at pH 4 : Activity at pH 5 ratio**

The activity at pH 4 of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2) and of variants according to the invention was determined using assay 2D as described herein.

10 The activity at pH 5 of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2) and of variants according to the invention was determined using assay 2A as described herein.

Activity at pH4 : Activity at pH5 ratio of variants according to the invention was determined as the ratio of

15 [Activity of the variant in Assay 2D (as described herein)] to [Activity of the variant in Assay 2A (as described herein)],

expressed as a percentage of the ratio of

[Activity on maltotriose of the reference polypeptide in Assay 2D (as described herein)] to [Activity on maltotriose of the reference polypeptide in Assay 2A (as described herein)].

20 The percentage thus obtained is the Activity at pH 4 : Activity at pH 5 ratio as listed in table 7 below.

An Activity at pH 4 : Activity at pH 5 ratio of more than 100% shows that the variant has an increased Activity at pH 4 : Activity at pH 5 ratio compared to the reference polypeptide.

25 Table 7 Activity at pH 4 : Activity at pH 5 ratio of variants according to the invention compared to the reference polypeptide. The Activity at pH 4 : Activity at pH 5 ratio of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID

NO: 2), was set at 100%. An Activity at pH 4 : Activity at pH 5 ratio of more than 100% shows that the variant has an increased Activity at pH 4 : Activity at pH 5 ratio compared to the reference polypeptide.

Variant#	Substitutions *)	Activity at pH 4 : Activity at pH 5 ratio of variant compared with reference polypeptide (set at 100%) (average of 2 measurements)
V11	W70Y, L282F and L225F	110%
V12	W70Y, L282I and L225F	109%
V27	W70Y, L282I, S133T and L225F	110%
V32	L225F and I14V	132%
V33	L225F and I15V	127%
V34	L225F and S358A	149%
V35	L225F and V124I	140%
V36	L225F, S358A and I14V	150%
V37	L225F, V124I and I15V	131%
V38	S200N, L225F and I14V	138%
V39	S200N, L225F and I15V	137%
V40	S200N, L225F and S358A	136%
V41	S200N, L225F and V124I	141%
V42	S200N, L225F, S358A and I14V	141%
V43	S200N, L225F, V124I and I15V	139%
V45	S200N, L282I, S133T and L225F	134%
V46	S200N, L282F, S133T, L225F and S358A	127%

*) W70Y and S200N means 2 changes: the amino acid at position 70 and the amino acid at position 200 are substituted. The W (Tryptophan) at position 70 is changed into a Y (Tyrosine) and that S (Serine) at position 200 is changed into a N (Asparagine), etc.

Example 7 Thermostability at pH 4

Thermostability at pH 4 of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2) and of variants according to the invention was determined using assays 2A and 2E as described herein.

5 Thermostability at pH 4 of variants according to the invention having an amino acid sequence according to SEQ ID NO: 2 including an amino acid change as indicated in table 8 below and of the reference polypeptide having an amino acid sequence as set out in SEQ ID NO: 2 was determined as described herein under Materials and Methods above. This done using Assay 2A, Assay 2C and “Exemplary determination and
10 calculation of thermostability of variant #” as described above, applied analogously to the ratio of

[Residual Activity of variant # in Assay 2E] : [Activity of variant # in Assay 2A],

expressed as a percentage of the ratio of

[Residual Activity of reference polypeptide in Assay 2E] : [Activity of reference
15 polypeptide in Assay 2A].

The percentage thus obtained is the Thermostability at pH 4 as listed in table 8 below.

A thermostability at pH 4 of more than 100% shows that the variant has an increased thermostability at pH 4 compared to the reference polypeptide.

20 Table 8 Thermostability at pH 4 of variants according to the invention compared to the reference polypeptide. The thermostability at pH 4 of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2), was set at 100%. A thermostability at pH 4 of more than 100% shows that the variant has an increased thermostability at pH 4 compared to the reference polypeptide.

Variant#	Substitutions *)	Thermostability at pH 4 of variant compared with reference polypeptide (set at 100%) (average of 2 measurements)
V01	W70Y and S200N	164%
V02	W70Y and L282F	141%
V03	W70Y and L282I	139%
V05	W70Y and L225F	132%
V06	W70Y and I14V	141%
V07	W70Y and I15V	134%
V08	W70Y and S358A	127%
V09	W70Y and V124I	142%
V10	W70Y, S200N and L225F	161%
V11	W70Y, L282F and L225F	152%
V12	W70Y, L282I and L225F	124%
V13	W70Y, S133T and L225F	143%
V14	W70Y, S200N and S358A	165%
V15	W70Y, L282F and S358A	144%
V16	W70Y, L282I and S358A	132%
V17	W70Y, S133T and S358A	148%
V18	W70Y, S200N and V124I	158%
V19	W70Y, L282F and V124I	150%
V20	W70Y, L282I and V124I	142%
V21	W70Y, S133T and V124I	160%
V22	W70Y, S200N and S133T	163%
V23	W70Y, L282F and S133T	150%
V25	W70Y, S200N, S133T and L225F	147%
V26	W70Y, L282F, S133T and L225F	145%
V27	W70Y, L282I, S133T and L225F	127%
V28	W70Y, S200N, S133T and S358A	161%
V29	W70Y, L282F, S133T and S358A	164%

V30	W70Y, S200N, S133T and V124I	171%
V38	S200N, L225F and I14V	132%
V39	S200N, L225F and I15V	129%
V40	S200N, L225F and S358A	127%
V41	S200N, L225F and V124I	127%
V43	S200N, L225F, V124I and I15V	128%
V45	S200N, L282I, S133T and L225F	136%
V46	S200N, L282F, S133T, L225F and S358A	152%

*) W70Y and S200N means 2 changes: the amino acid at position 70 and the amino acid at position 200 are substituted. The W (Tryptophan) at position 70 is changed into a Y (Tyrosine) and that S (Serine) at position 200 is changed into a N (Asparagine), etc.

5 **Example 8 Activity at pH 7 : Activity at pH 5 ratio**

The activity at pH 7 of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2) and of variants according to the invention was determined using assay 2G as described herein.

10 The activity at pH 5 of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2) and of variants according to the invention was determined using assay 2A as described herein.

Activity at pH 7 : Activity at pH 5 ratio of variants according to the invention was determined as the ratio of
[Activity of the variant in Assay 2G (as described herein)] to [Activity of the variant in Assay 2A (as described herein)],

5 expressed as a percentage of the ratio of
[Activity on maltotriose of the reference polypeptide in Assay 2G (as described herein)]
to [Activity on maltotriose of the reference polypeptide in Assay 2A (as described herein)].

10 The percentage thus obtained is the Activity at pH 7 : Activity at pH 5 ratio as listed in
table 9 below.

An Activity at pH 7 : Activity at pH 5 ratio of more than 100% shows that the variant has an increased Activity at pH 7 : Activity at pH 5 ratio compared to the reference polypeptide.

15 Table 9 Activity at pH 7 : Activity at pH 5 ratio of variants according to the invention compared to the reference polypeptide. The Activity at pH 7 : Activity at pH 5 ratio of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2), was set at 100%. An Activity at pH 7 : Activity at pH 5 ratio of more than 100% shows that the variant has an increased Activity at pH 7 : Activity at pH 5 ratio compared
20 to the reference polypeptide.

Variant#	Substitutions *)	Activity at pH 7 : Activity at pH 5 ratio of variant compared with reference polypeptide (set at 100%) (average of 2 measurements)
V01	W70Y and S200N	134%
V03	W70Y and L282I	150%
V05	W70Y and L225F	158%
V06	W70Y and I14V	143%
V07	W70Y and I15V	140%
V08	W70Y and S358A	169%
V09	W70Y and V124I	141%
V10	W70Y, S200N and L225F	153%
V11	W70Y, L282F and L225F	127%
V12	W70Y, L282I and L225F	166%
V13	W70Y, S133T and L225F	172%
V14	W70Y, S200N and S358A	161%
V15	W70Y, L282F and S358A	138%
V16	W70Y, L282I and S358A	181%
V17	W70Y, S133T and S358A	174%
V18	W70Y, S200N and V124I	135%
V19	W70Y, L282F and V124I	120%
V20	W70Y, L282I and V124I	158%
V21	W70Y, S133T and V124I	148%
V22	W70Y, S200N and S133T	154%
V25	W70Y, S200N, S133T and L225F	152%
V26	W70Y, L282F, S133T and L225F	125%
V27	W70Y, L282I, S133T and L225F	160%
V28	W70Y, S200N, S133T and S358A	166%
V29	W70Y, L282F, S133T and S358A	140%
V30	W70Y, S200N, S133T and V124I	147%

V34	L225F and S358A	120%
V45	S200N, L282I, S133T and L225F	145%
V46	S200N, L282F, S133T, L225F and S358A	172%

*) W70Y and S200N means 2 changes: the amino acid at position 70 and the amino acid at position 200 are substituted. The W (Tryptophan) at position 70 is changed into a Y (Tyrosine) and that S (Serine) at position 200 is changed into a N (Asparagine), etc.

5 Example 9 Sucrose tolerance at pH 7

Sucrose tolerance of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2) and of variants according to the invention was determined at pH 7 using assays 2A and 2H as described herein.

10 Sucrose tolerance at pH 7 of variants according to the invention having an amino acid sequence according to SEQ ID NO: 2 including the substitutions as indicated in table 10 below and of the reference alpha amylase polypeptide having an amino acid sequence as set out in SEQ ID NO: 2 was determined as described herein under Materials and Methods above (Assay 2A, Assay 2H and “Exemplary determination and calculation of sucrose tolerance of variant #” applied analogously)

15 The sucrose tolerance at pH 7 of variants according to the invention was determined as the ratio of
 [Activity of the variant in Assay 2H (as described herein)] to [Activity of the variant in Assay 2A (as described herein)],
 expressed as a percentage of the ratio of
 20 [Activity of the reference polypeptide in Assay 2H (as described herein)] to [Activity of the reference polypeptide in Assay 2A (as described herein)].

The percentage thus obtained is the Sucrose tolerance at pH 7 as listed in Table 10 below.

25 A sucrose tolerance of more than 100% shows that the variant has an increased sucrose tolerance compared to the reference polypeptide.

Table 10 Sucrose tolerance at pH 7 of variants according to the invention compared to the reference polypeptide. The sucrose tolerance at pH 7 of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2), was set at 100%. A sucrose tolerance at pH 7 of more than 100% shows that the variant has an increased sucrose tolerance compared to the reference polypeptide.

Variant#	Substitutions *)	Sucrose tolerance at pH 7 of variant compared with sucrose tolerance of reference polypeptide (set at 100%) (average of 2 measurements)
V01	W70Y and S200N	150%
V05	W70Y and L225F	212%
V07	W70Y and I15V	144%
V08	W70Y and S358A	166%
V09	W70Y and V124I	148%
V10	W70Y, S200N and L225F	204%
V11	W70Y, L282F and L225F	203%
V12	W70Y, L282I and L225F	198%
V13	W70Y, S133T and L225F	225%
V14	W70Y, S200N and S358A	184%
V15	W70Y, L282F and S358A	151%
V16	W70Y, L282I and S358A	151%
V17	W70Y, S133T and S358A	165%
V21	W70Y, S133T and V124I	156%
V22	W70Y, S200N and S133T	156%
V25	W70Y, S200N, S133T and L225F	209%
V26	W70Y, L282F, S133T and L225F	199%
V27	W70Y, L282I, S133T and L225F	186%
V28	W70Y, S200N, S133T and S358A	187%
V29	W70Y, L282F, S133T and S358A	151%
V30	W70Y, S200N, S133T and V124I	142%

V32	L225F and I14V	151%
V33	L225F and I15V	167%
V34	L225F and S358A	187%
V35	L225F and V124I	160%
V36	L225F, S358A and I14V	190%
V37	L225F, V124I and I15V	181%
V38	S200N, L225F and I14V	165%
V39	S200N, L225F and I15V	165%
V40	S200N, L225F and S358A	177%
V41	S200N, L225F and V124I	163%
V42	S200N, L225F, S358A and I14V	159%
V43	S200N, L225F, V124I and I15V	185%
V45	S200N, L282I, S133T and L225F	157%
V46	S200N, L282F, S133T, L225F and S358A	302%

*) W70Y and S200N means 2 changes: the amino acid at position 70 and the amino acid at position 200 are substituted. The W (Tryptophan) at position 70 is changed into a Y (Tyrosine) and that S (Serine) at position 200 is changed into a N (Asparagine), etc.

5 Example 10 Thermostability at pH 7

Thermostability at pH 7 of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2) and of variants according to the invention was determined using assays 2A and 2J as described herein.

Thermostability at pH 7 of variants according to the invention having an amino acid sequence according to SEQ ID NO: 2 including an amino acid change as indicated in table 11 below and of the reference polypeptide having an amino acid sequence as set out in SEQ ID NO: 2 was determined as described herein under Materials and Methods above. This done using Assay 2A, Assay 2J and “Exemplary determination and calculation of thermostability of variant #” as described above, applied analogously to the ratio of

[Residual Activity of variant # in Assay 2J] : [Activity of variant # in Assay 2A],

expressed as a percentage of the ratio of

[Residual Activity of reference polypeptide in Assay 2J] : [Activity of reference polypeptide in Assay 2A].

The percentage thus obtained is the Thermostability at pH 7 as listed in table 11.

A thermostability at pH 7 of more than 100% shows that the variant has an increased thermostability at pH 7 compared to the reference polypeptide.

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Table 11 Thermostability at pH 7 of variants according to the invention compared to the reference polypeptide. The thermostability at pH 7 of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2), was set at 100%. A thermostability at pH 7 of more than 100% shows that the variant has an increased thermostability at pH 7 compared to the reference polypeptide.

20

Variant#	Substitutions *)	Thermostability at pH 7 of variant compared with reference polypeptide (set at 100%)
		(average of 2 measurements)
V01	W70Y and S200N	537%
V02	W70Y and L282F	501%
V03	W70Y and L282I	518%
V05	W70Y and L225F	366%
V06	W70Y and I14V	438%
V07	W70Y and I15V	446%
V08	W70Y and S358A	449%
V09	W70Y and V124I	446%
V10	W70Y, S200N and L225F	433%
V11	W70Y, L282F and L225F	397%
V12	W70Y, L282I and L225F	409%
V13	W70Y, S133T and L225F	398%
V14	W70Y, S200N and S358A	512%
V15	W70Y, L282F and S358A	476%
V16	W70Y, L282I and S358A	465%
V17	W70Y, S133T and S358A	434%
V18	W70Y, S200N and V124I	464%
V19	W70Y, L282F and V124I	509%
V20	W70Y, L282I and V124I	518%
V21	W70Y, S133T and V124I	511%
V22	W70Y, S200N and S133T	506%
V23	W70Y, L282F and S133T	481%
V25	W70Y, S200N, S133T and L225F	460%
V26	W70Y, L282F, S133T and L225F	420%
V27	W70Y, L282I, S133T and L225F	430%

V28	W70Y, S200N, S133T and S358A	533%
V29	W70Y, L282F, S133T and S358A	487%
V30	W70Y, S200N, S133T and V124I	486%
V38	S200N, L225F and I14V	166%
V40	S200N, L225F and S358A	185%
V41	S200N, L225F and V124I	170%
V42	S200N, L225F, S358A and I14V	187%
V45	S200N, L282I, S133T and L225F	298%
V46	S200N, L282F, S133T, L225F and S358A	375%

*) W70Y and S200N means 2 changes: the amino acid at position 70 and the amino acid at position 200 are substituted. The W (Tryptophan) at position 70 is changed into a Y (Tyrosine) and that S (Serine) at position 200 is changed into a N (Asparagine), etc.

5

Example 11 Activity at 60°C : Activity at 37°C ratio.

Activity at 60°C (amount of glucose released per minute) of variants according to the invention compared to the reference polypeptide was determined using Assay 2P.

10 Activity at 37°C (amount of glucose released per minute) ratio of variants according to the invention compared to the reference polypeptide was determined using Assay 2A.

Activity at 60°C (amount of glucose released per minute) : Activity at 37°C (amount of glucose released per minute) ratio of variants according to the invention was determined as the ratio of

[Activity of the variant in Assay 2P (as described herein)] to [Activity of the variant in Assay 2A (as described herein)],
 expressed as a percentage of the ratio of
 [Activity of the reference polypeptide in Assay 2P (as described herein)] to [Activity of
 5 the reference polypeptide in Assay 2A (as described herein)].

The percentage thus obtained is the Activity at 60°C : Activity at 37°C ratio as listed in table 12 below.

10 An Activity at 60°C : Activity at 37°C ratio of more than 100% shows that the variant has an increased the Activity at 60°C : Activity at 37°C compared to the reference polypeptide.

15 Table 12 Activity at 60°C : Activity at 37°C ratio of variants according to the invention compared to the reference polypeptide. The Activity at pH 60°C : Activity at 37°C ratio of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2), was set at 100%. An Activity at pH 60°C : Activity at 37°C ratio of more than 100% shows that the variant has an increased Activity at 60°C : Activity at 37°C ratio compared to the reference polypeptide.

Variant#	Substitutions *)	Activity at 60°C : Activity at 37°C ratio of variant compared with reference polypeptide (set at 100%) (average of 2 measurements)
V01	W70Y and S200N	181%
V02	W70Y and L282F	165%
V03	W70Y and L282I	160%
V05	W70Y and L225F	209%
V06	W70Y and I14V	171%
V07	W70Y and I15V	156%
V08	W70Y and S358A	184%

V09	W70Y and V124I	172%
V10	W70Y, S200N and L225F	206%
V11	W70Y, L282F and L225F	181%
V12	W70Y, L282I and L225F	180%
V13	W70Y, S133T and L225F	193%
V14	W70Y, S200N and S358A	173%
V15	W70Y, L282F and S358A	157%
V16	W70Y, L282I and S358A	162%
V17	W70Y, S133T and S358A	154%
V18	W70Y, S200N and V124I	154%
V19	W70Y, L282F and V124I	164%
V20	W70Y, L282I and V124I	140%
V21	W70Y, S133T and V124I	143%
V22	W70Y, S200N and S133T	158%
V23	W70Y, L282F and S133T	137%
V25	W70Y, S200N, S133T and L225F	167%
V26	W70Y, L282F, S133T and L225F	148%
V27	W70Y, L282I, S133T and L225F	153%
V28	W70Y, S200N, S133T and S358A	166%
V29	W70Y, L282F, S133T and S358A	156%
V30	W70Y, S200N, S133T and V124I	159%
V32	L225F and I14V	235%
V33	L225F and I15V	199%
V34	L225F and S358A	307%
V35	L225F and V124I	257%
V36	L225F, S358A and I14V	305%
V37	L225F, V124I and I15V	291%
V38	S200N, L225F and I14V	245%
V39	S200N, L225F and I15V	199%
V40	S200N, L225F and S358A	270%
V41	S200N, L225F and V124I	263%
V42	S200N, L225F, S358A and I14V	338%
V43	S200N, L225F, V124I and I15V	257%

V45	S200N, L282I, S133T and L225F	215%
V46	S200N, L282F, S133T, L225F and S358A	207%

*) W70Y and S200N means 2 changes: the amino acid at position 70 and the amino acid at position 200 are substituted. The W (Tryptophan) at position 70 is changed into a Y (Tyrosine) and that S (Serine) at position 200 is changed into a N (Asparagine), etc.

5

Example 12 - Activity at 80°C : Activity at 37°C ratio.

Activity at 80°C (amount of glucose released per minute) of variants according to the invention compared to the reference polypeptide was determined using Assay 2Q.

Activity at 37°C (amount of glucose released per minute) ratio of variants according to the invention compared to the reference polypeptide was determined using Assay 2A.

10

Activity at 80°C (amount of glucose released per minute) : Activity at 37°C (amount of glucose released per minute) ratio of variants according to the invention was determined as the ratio of

[Activity of the variant in Assay 2Q (as described herein)] to [Activity of the variant in Assay 2A (as described herein)],

15

expressed as a percentage of the ratio of

[Activity of the reference polypeptide in Assay 2Q (as described herein)] to [Activity of the reference polypeptide in Assay 2A (as described herein)].

The percentage thus obtained is the Activity at 60°C : Activity at 37°C ratio as listed in table 13 below.

20

An Activity at 80°C : Activity at 37°C ratio of more than 100% shows that the variant has an increased the Activity at 80°C : Activity at 37°C compared to the reference polypeptide.

25

Table 13 Activity at 80°C : Activity at 37°C ratio of variants according to the invention compared to the reference polypeptide. The Activity at pH 80°C : Activity at 37°C ratio of the reference polypeptide (polypeptide having an amino acid sequence as set out in SEQ ID NO: 2), was set at 100%. An Activity at pH 80°C : Activity at 37°C ratio of more

than 100% shows that the variant has an increased Activity at 80°C : Activity at 37°C ratio compared to the reference polypeptide.

Variant#	Substitutions *)	Activity at 80°C : Activity at 37°C ratio of variant compared with reference polypeptide (set at 100%) (average of 2 measurements)
V01	W70Y and S200N	318%
V02	W70Y and L282F	290%
V03	W70Y and L282I	259%
V05	W70Y and L225F	482%
V06	W70Y and I14V	317%
V07	W70Y and I15V	285%
V08	W70Y and S358A	358%
V09	W70Y and V124I	323%
V10	W70Y, S200N and L225F	506%
V11	W70Y, L282F and L225F	399%
V12	W70Y, L282I and L225F	382%
V13	W70Y, S133T and L225F	459%
V14	W70Y, S200N and S358A	346%
V15	W70Y, L282F and S358A	324%
V16	W70Y, L282I and S358A	300%
V17	W70Y, S133T and S358A	302%
V18	W70Y, S200N and V124I	315%
V19	W70Y, L282F and V124I	333%
V20	W70Y, L282I and V124I	269%
V21	W70Y, S133T and V124I	291%
V22	W70Y, S200N and S133T	303%
V23	W70Y, L282F and S133T	296%
V25	W70Y, S200N, S133T and L225F	438%
V26	W70Y, L282F, S133T and L225F	325%

V27	W70Y, L282I, S133T and L225F	356%
V28	W70Y, S200N, S133T and S358A	330%
V29	W70Y, L282F, S133T and S358A	317%
V30	W70Y, S200N, S133T and V124I	309%
V32	L225F and I14V	445%
V33	L225F and I15V	336%
V34	L225F and S358A	740%
V35	L225F and V124I	601%
V36	L225F, S358A and I14V	731%
V37	L225F, V124I and I15V	443%
V38	S200N, L225F and I14V	438%
V39	S200N, L225F and I15V	354%
V40	S200N, L225F and S358A	621%
V41	S200N, L225F and V124I	538%
V42	S200N, L225F, S358A and I14V	695%
V43	S200N, L225F, V124I and I15V	493%
V45	S200N, L282I, S133T and L225F	437%
V46	S200N, L282F, S133T, L225F and S358A	625%

*) W70Y and S200N means 2 changes: the amino acid at position 70 and the amino acid at position 200 are substituted. The W (Tryptophan) at position 70 is changed into a Y (Tyrosine) and that S (Serine) at position 200 is changed into a N (Asparagine), etc.

CLAIMS

- 5 1. A variant polypeptide having alpha-amylase activity, wherein the variant has an amino acid sequence having at least 70% identity with the polypeptide sequence as set out in SEQ ID NO: 2 which, when aligned with the sequence as set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 200,
10 225, 282, 358, said positions being defined with reference to SEQ ID NO: 2; and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha amylase activity as set out in SEQ ID NO: 2.
- 15 2. A variant polypeptide having alpha-amylase activity according to claim 1, wherein the variant has an amino acid sequence which, when aligned with the alpha-amylase comprising the sequence set out in SEQ ID NO: 2, comprises a substitution of an amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue
20 corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having alpha-amylase activity.
- 25 3. The variant according to claim 2, wherein the reference polypeptide having alpha-amylase activity is the polypeptide as set out in SEQ ID NO: 2.
- 30 4. The variant according to any one of claims 1 to 3, wherein the variant has an amino acid sequence which comprises the amino acid substitution W70Y and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2.

5. The variant according to any one of claims 1 to 4, wherein the variant has an amino acid sequence which comprises one of the following sets of substitutions:

W70Y and S200N;
W70Y and L282F;
W70Y and L282I;
W70Y and S133T;
W70Y and L225F;
W70Y and I14V;
W70Y and I15V;
W70Y and S358A;
W70Y and V124I;
W70Y, S200N and L225F;
W70Y, L282F and L225F;
W70Y, L282I and L225F;
W70Y, S133T and L225F;
W70Y, S200N and S358A;
W70Y, L282F and S358A;
W70Y, L282I and S358A;
W70Y, S133T and S358A;
W70Y, S200N and V124I;
W70Y, L282F and V124I;
W70Y, L282I and V124I;
W70Y, S133T and V124I;
W70Y, S200N and S133T;
W70Y, L282F and S133T;
W70Y, L282I and S133T;
W70Y, S200N, S133T and L225F;
W70Y, L282F, S133T and L225F;
W70Y, L282I, S133T and L225F;
W70Y, S200N, S133T and S358A;
W70Y, L282F, S133T and S358A;
W70Y, S200N, S133T and V124I;

W70Y, L282F, S133T and V124I;

said positions being defined with reference to SEQ ID NO: 2.

6. The variant according to any one of claims 1 to 5, wherein the one or more altered properties include an increased thermostability.

5

7. The variant according to any one of claims 1 to 5, wherein the one or more altered properties include an increased sucrose tolerance.

8. The variant according to any one of claims 1 to 5, wherein the one or more altered properties include an increased an increased Activity at pH4 : Activity at pH5 ratio and/or an increased Activity at pH7 : Activity at pH5 ratio.

10

9. A nucleic acid sequence encoding a variant polypeptide according to any one of the preceding claims.

15

10. A nucleic acid construct comprising the nucleic acid sequence of claim 9 operably linked to one or more control sequences capable of directing the expression of an alpha-amylase in a suitable expression host.

20

11. A recombinant expression vector comprising the nucleic acid construct of claim 10.

12. A recombinant host cell comprising the expression vector of claim 11.

25

13. A method for producing an alpha-amylase comprising cultivating the host cell of claim 12 under conditions conducive to production of the alpha-amylase and recovering the alpha-amylase.

14. A composition comprising the variant polypeptide according to any one of claims 1 to 8 or obtainable by a method according to claim 13 and one or more components selected from the group consisting of milk, gluten, granulated fat, an additional enzyme, an amino acid, a salt, oxidants,

30

reducing agents, emulsifiers, gums, flavours, acids, starch, modified starch, humectants and preservatives.

5 15. A pre-mix comprising flour and the variant polypeptide according to any one of claims 1 to 8 or obtainable by the method according to claim 13.

10 16. Use of a variant polypeptide according to any one of claims 1 to 8 or of the composition according to claim 14 or of the pre-mix according to claim 15 in the preparation of a dough and/or a baked product.

17. A dough comprising the variant polypeptide according to any one of claims 1 to 8 or of the composition according to claim 14 or of the pre-mix according to claim 15.

15 18. A process for the production of a baked product, which method comprises baking the dough according to claim 17.

19. A baked product obtainable by the process according to claim 18 or by the use according to claim 16.

20 20. A method of producing an alpha-amylase polypeptide variant, which method comprises:

- 25
- a) selecting an alpha-amylase polypeptide;
 - b) substituting at least one amino acid residue corresponding to amino acid 70 and at least one further substitution of an amino acid residue corresponding to any of amino acids 14, 15, 124, 133, 225, 200, 282, 358, said positions being defined with reference to SEQ ID NO: 2;
 - c) optionally substituting one or more further amino acids as defined in b);
 - d) preparing the variant resulting from steps a)-c);
 - e) determining a property of the variant; and
- 30

- f) selecting a variant having an altered property in comparison to the polypeptide of a), thereby to produce an alpha-amylase polypeptide variant.

- 5 21. Use of a variant polypeptide according to any one of claims 1 to 8 or of the composition according to claim 14 or of the pre-mix according to claim 15 in the food industry.

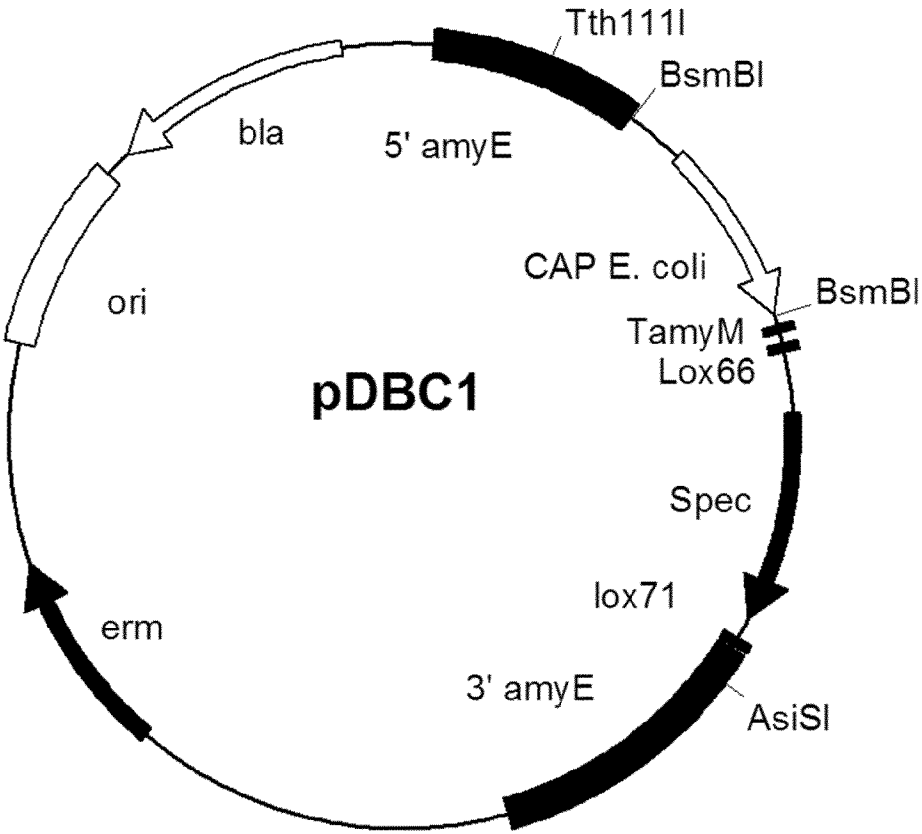


Fig. 1

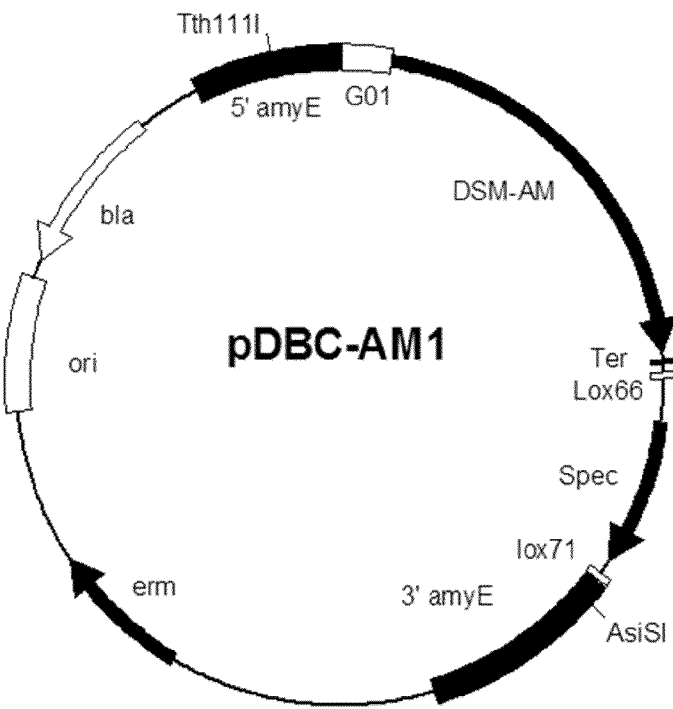


Fig. 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/069612

A. CLASSIFICATION OF SUBJECT MATTER
INV. A21D8/04 C12N9/26 C11D3/386
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A21D C12N C11D

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

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

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2014/131842 A1 (DSM IP ASSETS BV [NL]) 4 September 2014 (2014-09-04) Whole doc., in particular sequences and mutation sets encompassing W70Y mutation. -----	1-21
A	US 8 426 182 B1 (PARENICOVA LUCIE [NL]) 23 April 2013 (2013-04-23) Whole doc., in particular Seq. ID No.1; examples 7, 8 -----	1-21
A	WO 2008/148845 A2 (NOVOZYMES AS [DK]; BEIER LARS [DK]; FRIIS ESBEN PETER [DK]; LUNDQVIST) 11 December 2008 (2008-12-11) Whole do., in particular I15T variant (e.g. Table 1; claim 8) ----- -/-	1-21



Further documents are listed in the continuation of Box C.



See patent family annex.

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

24 September 2015

Date of mailing of the international search report

06/10/2015

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/069612

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2011/058105 A1 (NOVOZYMES AS [DK]; FREDERIKSEN ANNE METTE BHATIA [DK]; BEIER LARS [DK]) 19 May 2011 (2011-05-19) Sequence variation equiv. to pos. 358 (S358P) - Table 6 -----	1-21
A	DATABASE UniProt [Online] 19 February 2014 (2014-02-19), "SubName: Full=Alpha amylase {ECO:0000313 EMBL:CDK35218.1}";", XP002744948, retrieved from EBI accession no. UNIPROT:V6DNK0 Database accession no. V6DNK0 Sequence; sequence -----	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2015/069612

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014131842 A1	04-09-2014	CA 2902996 A1	04-09-2014
		WO 2014131842 A1	04-09-2014

US 8426182 B1	23-04-2013	AR 089840 A1	24-09-2014
		AU 2013214397 A1	24-07-2014
		CA 2861148 A1	08-08-2013
		CL 2014002030 A1	10-04-2015
		CN 104220593 A	17-12-2014
		DK 2620496 T3	07-09-2015
		EA 201400850 A1	30-12-2014
		EP 2620496 A1	31-07-2013
		EP 2809781 A1	10-12-2014
		US 8426182 B1	23-04-2013
		US 2014377407 A1	25-12-2014
		WO 2013113665 A1	08-08-2013

WO 2008148845 A2	11-12-2008	AU 2008258554 A1	11-12-2008
		CA 2689972 A1	11-12-2008
		DK 2164958 T3	24-11-2014
		DK 2527430 T3	13-04-2015
		EP 2164958 A2	24-03-2010
		EP 2527430 A2	28-11-2012
		US 2010215802 A1	26-08-2010
		WO 2008148845 A2	11-12-2008

WO 2011058105 A1	19-05-2011	CN 102639687 A	15-08-2012
		DK 2499227 T3	13-07-2015
		EP 2499227 A1	19-09-2012
		ES 2539726 T3	03-07-2015
		US 2012225164 A1	06-09-2012
		WO 2011058105 A1	19-05-2011
