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(54) **MUTANTS D' α -AMYLASE**
(54) **α -AMYLASE MUTANTS**

(57) L'invention a trait à une variante d'une α -amylase de type termamyle mère, qui comporte des mutations dans deux, trois, quatre, cinq ou six régions/positions. Les variantes présentent une thermostabilité accrue à des pH acides et/ou à des concentrations faibles de Ca^{2+} (par rapport à la substance mère). L'invention a également trait à une structure d'ADN comportant une séquence d'ADN codant pour une variante d' α -amylase de l'invention, à un vecteur d'expression recombiné qui porte une structure d'ADN de l'invention, à une cellule qui est transformée au moyen d'une structure d'ADN de l'invention, à l'utilisation d'une variante d' α -amylase de l'invention à des fins de lavage et/ou de lavage de vaisselle, de désencollage de textiles, de liquéfaction d'amidon, à un additif de détergent comportant une variante d' α -amylase de l'invention, à une composition de détergent pour vaisselle à la main ou à la machine renfermant une variante d' α -amylase de l'invention, à un procédé servant à produire une variante d'une α -amylase de type termamyle mère, cette variante présentant une thermostabilité accrue à des pH acides et/ou à des concentrations faibles de Ca^{2+} (par rapport à la substance mère).

(57) The invention relates to a variant of a parent Termamyl-like α -amylase, comprising mutations in two, three, four, five or six regions/positions. The variants have increased thermostability at acidic pH and/or at low Ca^{2+} concentrations (relative to the parent). The invention also relates to a DNA construct comprising a DNA sequence encoding an α -amylase variant of the invention, a recombinant expression vector which carries a DNA construct of the invention, a cell which is transformed with a DNA construct of the invention, the use of an α -amylase variant of the invention for washing and/or dishwashing, textile desizing, starch liquefaction, a detergent additive comprising an α -amylase variant of the invention, a manual or automatic dishwashing detergent composition comprising an α -amylase variant of the invention, a method for generating a variant of a parent Termamyl-like α -amylase, which variant exhibits increased thermostability at acidic pH and/or at low Ca^{2+} concentrations (relative to the parent).

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(21) International Application Number: PCT/DK98/00444 (22) International Filing Date: 13 October 1998 (13.10.98) (30) Priority Data: 1172/97 13 October 1997 (13.10.97) DK (71) Applicant: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). (72) Inventors: SVENDSEN, Allan; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). BORCHERT, Torben, Vedel; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). BISGÅRD-FRANTZEN, Henrik; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). (74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
(54) Title: α -AMYLASE MUTANTS (57) Abstract The invention relates to a variant of a parent Termamyl-like α-amylase, comprising mutations in two, three, four, five or six regions/positions. The variants have increased thermostability at acidic pH and/or at low Ca^{2+} concentrations (relative to the parent). The invention also relates to a DNA construct comprising a DNA sequence encoding an α-amylase variant of the invention, a recombinant expression vector which carries a DNA construct of the invention, a cell which is transformed with a DNA construct of the invention, the use of an α-amylase variant of the invention for washing and/or dishwashing, textile desizing, starch liquefaction, a detergent additive comprising an α-amylase variant of the invention, a manual or automatic dishwashing detergent composition comprising an α-amylase variant of the invention, a method for generating a variant of a parent Termamyl-like α-amylase, which variant exhibits increased thermostability at acidic pH and/or at low Ca^{2+} concentrations (relative to the parent).		

Title: α -amylase mutants

FIELD OF THE INVENTION

5 The present invention relates, *inter alia*, to novel variants (mutants) of parent Termamyl-like α -amylases, notably variants exhibiting increased thermostability at acidic pH and/or at low Ca^{2+} concentrations (relative to the parent) which are advantageous with respect to applications of the variants in,
10 industrial starch processing particularly (e.g. starch liquefaction or saccharification).

BACKGROUND OF THE INVENTION

α -Amylases (α -1,4-glucan-4-glucanohydrolases, EC 3.2.1.1)
15 constitute a group of enzymes which catalyze hydrolysis of starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides.

There is a very extensive body of patent and scientific literature relating to this industrially very important class of
20 enzymes. A number of α -amylase such as Termamyl-like α -amylases variants are known from e.g. WO 90/11352, WO 95/10603, WO 95/26397, WO 96/23873 and WO 96/23874.

Among more recent disclosures relating to α -amylases, WO 96/23874 provides three-dimensional, X-ray crystal structural
25 data for a Termamyl-like α -amylase which consists of the 300 N-terminal amino acid residues of the *B. amyloliquefaciens* α -amylase and amino acids 301-483 of the C-terminal end of the *B. licheniformis* α -amylase comprising the amino acid sequence (the latter being available commercially under the tradename
30 TermamylTM), and which is thus closely related to the industrially important *Bacillus* α -amylases (which in the present context are embraced within the meaning of the term "Termamyl-like α -amylases", and which include, *inter alia*, the *B. licheniformis*, *B. amyloliquefaciens* and *B. stearothermophilus*
35 α -amylases). WO 96/23874 further describes methodology for

designing, on the basis of an analysis of the structure of a parent Termamyl-like α -amylase, variants of the parent Termamyl-like α -amylase which exhibit altered properties relative to the parent.

5 WO 95/35382 (Gist Brocades B.V.) concerns amylolytic enzymes derived from *B. licheniformis* with improved properties allowing reduction of the Ca^{2+} concentration under application without a loss of performance of the enzyme. The amylolytic enzyme comprises one or more amino acid changes at positions selected
10 from the group of 104, 128, 187, 188 of the *B. licheniformis* α -amylase sequence.

WO 96/23873 (Novo Nordisk) discloses Termamyl-like α -amylase variants which have increased thermostability obtained by pairwise deletion in the region R181*, G182*, T183* and G184* of
15 the sequence shown in SEQ ID NO: 1 herein.

BRIEF DISCLOSURE OF THE INVENTION

The present invention relates to novel α -amylolytic variants (mutants) of a Termamyl-like α -amylase, in particular variants
20 exhibiting increased thermostability (relative to the parent) which are advantageous in connection with the industrial processing of starch (starch liquefaction, saccharification and the like).

The inventors have surprisingly found out that in case of
25 combining two, three, four, five or six mutations (will be described below), the thermostability of Termamyl-like α -amylases is increased at acidic pH and/or at low Ca^{2+} concentration in comparison to single mutations, such as the mutation disclosed in WO 96/23873 (Novo Nordisk), i.e. pairwise
30 deletion in the region R181*, G182*, T183* and G184* of the sequence shown in SEQ ID NO: 1 herein.

The invention further relates to DNA constructs encoding variants of the invention, to composition comprising variants of the invention, to methods for preparing variants of the
35 invention, and to the use of variants and compositions of the invention, alone or in combination with other α -amylolytic

enzymes, in various industrial processes, e.g., starch liquefaction.

BRIEF DESCRIPTION OF THE DRAWING

5 Figure 1 is an alignment of the amino acid sequences of six parent Termamyl-like α -amylases in the context of the invention. The numbers on the Extreme left designate the respective amino acid sequences as follows:

- 1: SEQ ID NO: 2,
- 10 2: Kaoamyl,
- 3: SEQ ID NO: 1,
- 4: SEQ ID NO: 5,
- 5: SEQ ID NO: 4,
- 6: SEQ ID NO: 3.

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DETAILED DISCLOSURE OF THE INVENTION

The Termamyl-like α -amylase

It is well known that a number of α -amylases produced by
20 *Bacillus* spp. are highly homologous on the amino acid level. For instance, the *B. licheniformis* α -amylase comprising the amino acid sequence shown in SEQ ID NO: 4 (commercially available as TermamylTM) has been found to be about 89% homologous with the *B. amyloliquefaciens* α -amylase comprising the amino acid sequence
25 shown in SEQ ID NO: 5 and about 79% homologous with the *B. stearothermophilus* α -amylase comprising the amino acid sequence shown in SEQ ID NO: 3. Further homologous α -amylases include an α -amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described
30 in detail in WO 95/26397, and the α -amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31.

Still further homologous α -amylases include the α -amylase produced by the *B. licheniformis* strain described in EP 0252666
35 (ATCC 27811), and the α -amylases identified in WO 91/00353 and

WO 94/18314. Other commercial Termamyl-like *B. licheniformis* α -amylases are OptithermTM and TakathermTM (available from Solvay), MaxamylTM (available from Gist-brocades/Genencor), Spezym AATM and Spezyme Delta AATM (available from Genencor), and
5 KeistaseTM (available from Daiwa).

Because of the substantial homology found between these α -amylases, they are considered to belong to the same class of α -amylases, namely the class of "Termamyl-like α -amylases".

Accordingly, in the present context, the term "Termamyl-like
10 α -amylase" is intended to indicate an α -amylase which, at the amino acid level, exhibits a substantial homology to TermamylTM, i.e. the *B. licheniformis* α -amylase having the amino acid sequence shown in SEQ ID NO: 4 herein. In other words, a Termamyl-like α -amylase is an α -amylase which has the amino acid
15 sequence shown in SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 or 8 herein, and the amino acid sequence shown in SEQ ID NO: 1 of WO 95/26397 (the same as the amino acid sequence shown as SEQ ID NO: 7 herein) or in SEQ ID NO: 2 of WO 95/26397 (the same as the amino acid sequence shown as SEQ ID NO: 8 herein) or in Tsukamoto et
20 al., 1988, (which amino acid sequence is shown in SEQ ID NO: 6 herein) or i) which displays at least 60%, preferred at least 70%, more preferred at least 75%, even more preferred at least 80%, especially at least 85%, especially preferred at least 90%, even especially more preferred at least 95% homology with at
25 least one of said amino acid sequences shown in SEQ ID NOS 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 and/or ii) displays immunological cross-reactivity with an antibody raised against at least one of said α -amylases, and/or iii) is encoded by a DNA sequence which hybridizes to the DNA sequences encoding the
30 above-specified α -amylases which are apparent from SEQ ID NOS: 9, 10, 11, or 12 of the present application (which encoding sequences encode the amino acid sequences shown in SEQ ID NOS: 1, 2, 3, 4 and 5 herein, respectively), from SEQ ID NO: 4 of WO 95/26397 (which DNA sequence, together with the stop codon TAA,

is shown in SEQ ID NO: 13 herein and encodes the amino acid sequence shown in SEQ ID NO: 8 herein) and from SEQ ID NO: 5 of WO 95/26397 (shown in SEQ ID NO: 14 herein), respectively.

In connection with property i), the "homology" may be determined by use of any conventional algorithm, preferably by use of the GAP programme from the GCG package version 7.3 (June 1993) using default values for GAP penalties, which is a GAP creation penalty of 3.0 and GAP extension penalty of 0.1, (Genetic Computer Group (1991) Programme Manual for the GCG Package, version 7, 575 Science Drive, Madison, Wisconsin, USA 53711).

A structural alignment between Termamyl and a Termamyl-like α -amylase may be used to identify equivalent/corresponding positions in other Termamyl-like α -amylases. One method of obtaining said structural alignment is to use the Pile Up programme from the GCG package using default values of gap penalties, i.e., a gap creation penalty of 3.0 and gap extension penalty of 0.1. Other structural alignment methods include the hydrophobic cluster analysis (Gaboriaud et al., (1987), FEBS LETTERS 224, pp. 149-155) and reverse threading (Huber, T ; Torda, AE, PROTEIN SCIENCE Vol. 7, No. 1 pp. 142-149 (1998)).

Property ii) of the α -amylase, i.e. the immunological cross reactivity, may be assayed using an antibody raised against, or reactive with, at least one epitope of the relevant Termamyl-like α -amylase. The antibody, which may either be monoclonal or polyclonal, may be produced by methods known in the art, e.g. as described by Hudson et al., Practical Immunology, Third edition (1989), Blackwell Scientific Publications. The immunological cross-reactivity may be determined using assays known in the art, examples of which are Western Blotting or radial immunodiffusion assay, e.g. as described by Hudson et al., 1989. In this respect, immunological cross-reactivity between the α -amylases having the amino acid sequences SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, or 8 respectively, have been found.

The oligonucleotide probe used in the characterization of the Termamyl-like α -amylase in accordance with property iii) above

may suitably be prepared on the basis of the full or partial nucleotide or amino acid sequence of the α -amylase in question.

Suitable conditions for testing hybridization involve presoaking in 5xSSC and prehybridizing for 1 hour at $\sim 40^{\circ}\text{C}$ in a solution of 20% formamide, 5xDenhardt's solution, 50mM sodium phosphate, pH 6.8, and 50mg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100mM ATP for 18 hours at $\sim 40^{\circ}\text{C}$, followed by three times washing of the filter in 2xSSC, 0.2% SDS at 40°C for 30 minutes (low stringency), preferred at 50°C (medium stringency), more preferably at 65°C (high stringency), even more preferably at $\sim 75^{\circ}\text{C}$ (very high stringency). More details about the hybridization method can be found in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989.

In the present context, "derived from" is intended not only to indicate an α -amylase produced or producible by a strain of the organism in question, but also an α -amylase encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. Finally, the term is intended to indicate an α -amylase which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the α -amylase in question. The term is also intended to indicate that the parent α -amylase may be a variant of a naturally occurring α -amylase, i.e. a variant which is the result of a modification (insertion, substitution, deletion) of one or more amino acid residues of the naturally occurring α -amylase.

Parent hybrid α -amylases

The parent α -amylase may be a hybrid α -amylase, i.e. an α -amylase which comprises a combination of partial amino acid sequences derived from at least two α -amylases.

The parent hybrid α -amylase may be one which on the basis of amino acid homology and/or immunological cross-reactivity and/or

DNA hybridization (as defined above) can be determined to belong to the Termamyl-like α -amylase family. In this case, the hybrid α -amylase is typically composed of at least one part of a Termamyl-like α -amylase and part(s) of one or more other α -
5 amylases selected from Termamyl-like α -amylases or non-Termamyl-like α -amylases of microbial (bacterial or fungal) and/or mammalian origin.

Thus, the parent hybrid α -amylase may comprise a combination of partial amino acid sequences deriving from at least two
10 Termamyl-like α -amylases, or from at least one Termamyl-like and at least one non-Termamyl-like bacterial α -amylase, or from at least one Termamyl-like and at least one fungal α -amylase. The Termamyl-like α -amylase from which a partial amino acid sequence derives may, e.g., be any of those specific Termamyl-like α -
15 amylases referred to herein.

For instance, the parent α -amylase may comprise a C-terminal part of an α -amylase derived from a strain of *B. licheniformis*, and a N-terminal part of an α -amylase derived from a strain of *B. amyloliquefaciens* or from a strain of *B. stearothermophilus*.
20 For instance, the parent α -amylase may comprise at least 430 amino acid residues of the C-terminal part of the *B. licheniformis* α -amylase, and may, e.g. comprise a) an amino acid segment corresponding to the 37 N-terminal amino acid residues of the *B. amyloliquefaciens* α -amylase having the amino acid
25 sequence shown in SEQ ID NO: 5 and an amino acid segment corresponding to the 445 C-terminal amino acid residues of the *B. licheniformis* α -amylase having the amino acid sequence shown in SEQ ID No. 4, or b) an amino acid segment corresponding to the 68 N-terminal amino acid residues of the *B. stearother-*
30 *mophilus* α -amylase having the amino acid sequence shown in SEQ ID NO: 3 and an amino acid segment corresponding to the 415 C-terminal amino acid residues of the *B. licheniformis* α -amylase having the amino acid sequence shown in SEQ ID NO: 4.

The non-Termamyl-like α -amylase may, e.g., be a fungal α -amylase, a mammalian or a plant α -amylase or a bacterial α -amylase (different from a Termamyl-like α -amylase). Specific examples of such α -amylases include the *Aspergillus oryzae* TAKA
5 α -amylase, the *A. niger* acid α -amylase, the *Bacillus subtilis* α -amylase, the porcine pancreatic α -amylase and a barley α -amylase. All of these α -amylases have elucidated structures which are markedly different from the structure of a typical Termamyl-like α -amylase as referred to herein.

10 The fungal α -amylases mentioned above, i.e. derived from *A. niger* and *A. oryzae*, are highly homologous on the amino acid level and generally considered to belong to the same family of α -amylases. The fungal α -amylase derived from *Aspergillus oryzae* is commercially available under the tradename Fungamyl™.

15 Furthermore, when a particular variant of a Termamyl-like α -amylase (variant of the invention) is referred to - in a conventional manner - by reference to modification (e.g. deletion or substitution) of specific amino acid residues in the amino acid sequence of a specific Termamyl-like α -amylase, it is
20 to be understood that variants of another Termamyl-like α -amylase modified in the equivalent position(s) (as determined from the best possible amino acid sequence alignment between the respective amino acid sequences) are encompassed thereby.

A preferred embodiment of a variant of the invention is one
25 derived from a *B. licheniformis* α -amylase (as parent Termamyl-like α -amylase), e.g. one of those referred to above, such as the *B. licheniformis* α -amylase having the amino acid sequence shown in SEQ ID NO: 4.

30 Construction of variants of the invention

The construction of the variant of interest may be accomplished by cultivating a microorganism comprising a DNA sequence encoding the variant under conditions which are conducive for producing the variant. The variant may then

subsequently be recovered from the resulting culture broth. This is described in detail further below.

Altered properties of variants of the invention

5 The following discusses the relationship between mutations which may be present in variants of the invention, and desirable alterations in properties (relative to those a parent, Termamyl-like α -amylase) which may result therefrom.

10 Increased thermostability at acidic pH and/or at low Ca^{2+} concentration

Mutations of particular relevance in relation to obtaining variants according to the invention having increased thermostability at acidic pH and/or at low Ca^{2+} concentration
15 include mutations at the following positions (relative to *B. licheniformis* α -amylase, SEQ ID NO: 4):

H156, N172, A181, N188, N190, H205, D207, A209, A210, E211, Q264, N265.

In the context of the invention the term "acidic pH" means a
20 pH below 7.0, especially below the pH range, in which industrial starch liquefaction processes are normally performed, which is between pH 5.5 and 6.2.

In the context of the present invention the term "low Calcium concentration" means concentrations below the normal level used
25 in industrial starch liquefaction. Normal concentrations vary depending of the concentration of free Ca^{2+} in the corn. Normally a dosage corresponding to 1mM (40ppm) is added which together with the level in corn gives between 40 and 60ppm free Ca^{2+} .

In the context of the invention the term "high temperatures"
30 means temperatures between 95°C and 160°C, especially the temperature range in which industrial starch liquefaction processes are normally performed, which is between 95°C and 105°C.

The inventors have now found that the thermostability at
35 acidic pH and/or at low Ca^{2+} concentration may be increased even more by combining certain mutations including the above

mentioned mutations and/or I201 with each other.

Said "certain" mutations are the following (relative to *B. licheniformis* α -amylase, SEQ ID NO: 4):

N190, D207, E211, Q264 and I201.

5 Said mutation may further be combined with deletions in one, preferably two or even three positions as described in WO 96/23873 (i.e. in positions R181, G182, T183, G184 in SEQ ID NO: 1 herein). According to the invention variants of a parent Termamyl-like α -amylase with α -amylase activity comprising
10 mutations in two, three, four, five or six of the above positions are contemplated.

It should be emphasised that not only the Termamyl-like α -amylases mentioned specifically below are contemplated. Also other commercial Termamyl-like α -amylases are contemplated. An
15 unexhaustive list of such α -amylases is the following:

α -amylases produced by the *B. licheniformis* strain described in EP 0252666 (ATCC 27811), and the α -amylases identified in WO 91/00353 and WO 94/18314. Other commercial Termamyl-like *B. licheniformis* α -amylases are OptithermTM and TakathermTM
20 (available from Solvay), MaxamylTM (available from Gist-brocades/Genencor), Spezym AATM Spezyme Delta AATM (available from Genencor), and KeistaseTM (available from Daiwa).

It may be mentioned here that amino acid residues, respectively, at positions corresponding to N190, I201, D207 and
25 E211, respectively, in SEQ ID NO: 4 constitute amino acid residues which are conserved in numerous Termamyl-like α -amylases. Thus, for example, the corresponding positions of these residues in the amino acid sequences of a number of Termamyl-like α -amylases which have already been mentioned (*vide*
30 *supra*) are as follows:

Table 1.

Termamyl-like α -amylase	N	I	D	E	Q

	<i>B. licheniformis</i> (SEQ ID NO: 4)	N190	I201	D207	E211	Q264
	<i>B. amyloliquefaciens</i> (SEQ ID NO: 5)	N190	V201	D207	E211	Q264
	<i>B. stearothermophilus</i> (SEQ ID NO: 3)	N193	L204	E210	E214	---
	<i>Bacillus</i> WO 95/26397 (SEQ ID NO: 2)	N195	V206	E212	E216	---
5	<i>Bacillus</i> WO 95/26397 (SEQ ID NO: 1)	N195	V206	E212	E216	---
	" <i>Bacillus</i> sp. #707" (SEQ ID NO: 6)	N195	I206	E212	E216	---

10 Mutations of these conserved amino acid residues are very important in relation to improving thermostability at acidic pH and/or at low calcium concentration, and the following mutations are of particular interest in this connection (with reference to the numbering of the *B. licheniformis* amino acid sequence shown in SEQ ID NO: 4).

15 Pair-wise amino acid deletions at positions corresponding to R179-G182 in SEQ ID NO: 5 corresponding to a gap in Seq ID NO: 4. when aligned with a numerous Termamyl-like α -amylases. Thus, for example, the corresponding positions of these residues in the amino acid sequences of a number of Termamyl-like α -amylases
20 which have already been mentioned (*vide supra*) are as follows:

Table 2.

	Termamyl-like α -amylase	Pair wise amino acid deletions among
25	<i>B. amyloliquefaciens</i> (SEQ ID No.5)	R176, G177, E178, G179
	<i>B. stearothermophilus</i> (SEQ ID No.3)	R179, G180, I181, G182
	<i>Bacillus</i> WO 95/26397 (SEQ ID No.2)	R181, G182, T183, G184
	<i>Bacillus</i> WO 95/26397 (SEQ ID No.1)	R181, G182, D183, G184
30	" <i>Bacillus</i> sp. #707" (SEQ ID No.6)	R181, G182, H183, G184

When using SEQ ID NO: 1 to SEQ ID NO: 6 as the backbone (i.e. as the parent Termamyl-like α -amylase) two, three, four,
35 five or six mutations may according to the invention be made in the following regions/positions to increase the thermostability at acidic pH and/or at low Ca^{2+} concentrations (relative to the parent):

(relative to Seq ID NO: 1 herein):

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12

- 1: R181*, G182*, T183*, G184*
- 2: N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 3: V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
- 4: E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 5 5: E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 6: K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
- (relative to SEQ ID NO: 2 herein):
- 1: R181*,G182*,D183*,G184*
- 2: N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 10 3: V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
- 4: E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 5: E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 6: K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
- (Relative to SEQ ID NO: 3 herein):
- 15 1: R179*,G180,I181*,G182*
- 2: N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 3: L204A,R,D,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
- 4: E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 5: E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 20 6: S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V
- Relative to SEQ ID NO: 4 herein):
- 1: Q178*,G179*
- 2: N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 3: I201A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V;
- 25 4: D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 5: E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 6: Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- (relative to SEQ ID NO: 5 herein):
- 1: R176*,G177*,E178,G179*
- 30 2: N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 3: V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
- 4: D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 5: E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 6: Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
- 35 (relative to SEQ ID NO: 6 herein):
- 1: R181*,G182*,H183*,G184*
- 2: N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

3: I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V;

4: E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

5: E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

6: K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V.

5 Comtemplated according to the present invention is combining three, four, five or six mutation.

Specific double mutations for backbone SEQ ID NO: 1 to SEQ ID NO: 6 are listed in the following.

10 Using SEQ ID NO: 1 as the backbone the following double mutantions resulting in the desired effect are comtemplated according to the invention:

-R181*/G182*/N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

-G182*/T183*/N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

-T183*/G184*/N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

15 -R181*/G182*/V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;

-G182*/T183*/V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;

-T183*/G184*/V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;

-R181*/G182*/E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

-G182*/T183*/E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

20 -T183*/G184*/E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

-R181*/G182*/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

-G182*/T183*/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

-T183*/G184*/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

-R181*/G182*/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

25 -G182*/T183*/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

-T183*/G184*/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

-N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V

/V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;

-N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V

30 /E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

-N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V

/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

-N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V

/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

35 -V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y

/E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

-V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y

/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

-V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y
 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 -E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 5 E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 -E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

Using SEQ ID NO: 2 as the backbone the following double
 10 mutantions resulting in the desired effect are contemplated
 according to the invention:

-R181*/G182*/N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -G182*/D183*/N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -D183*/G184*/N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 15 -R181*/G182*/V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
 -G182*/T183*/V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
 -T183*/G184*/V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
 -R181*/G182*/E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -G182*/T183*/E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 20 -T183*/G184*/E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -R181*/G182*/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -G182*/T183*/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -T183*/G184*/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -R181*/G182*/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 25 -G182*/T183*/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 -T183*/G184*/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 -N195 A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
 -N195 A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 30 /E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 35 -V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y
 /E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -V206 A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y

/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y
 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 -E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 5 /E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 -E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

10 Using SEQ ID NO. 3 as the backbone the following double
 mutantions resulting in the desired effect are contemplated
 according to the invention:

-R179*/G180*/N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -G180*/I181*/N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 15 -I181*/G182*/N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -R179*/G180*/L204A,R,D,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
 -G180*/I181*/L204A,R,D,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
 -I181*/G182*/L204A,R,D,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
 -R179*/G180*/E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 20 -G180*/I181*/E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -I181*/G182*/E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -R179*/G180*/E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -G180*/I181*/E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -I181*/G182*/E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 25 -R179*/G180*/S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
 -G180*/I181*/S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
 -I181*/G182*/S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
 -N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /L204A,R,D,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
 30 -N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 35 /S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
 -L204A,R,D,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V
 /E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

-L204A,R,D,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V
 /E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -L204A,R,D,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V
 /S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
 5 -E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
 -E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 10 /S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;

Using SEQ ID NO. 4 as the backbone the following double mutantions resulting in the desired effect are contemplated according to the invention:

-Q178*/G179*/N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 15 -Q178*/G179*/I201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -Q178*/G179*/D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -Q178*/G179*/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -R179*/G180*/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -N190/I201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 20 -N190/D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -N190/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -N190/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -I201/D207A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -I201/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 25 -I201/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -D207/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -D207/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -E211/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

Using SEQ ID NO: 5 as the backbone the following double mutantions resulting in the desired effect are contemplated according to the invention:

-R176*/G177*/N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -G177*/E178*/N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -E178*/G179*/N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 35 -R176*/G177*/V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
 -G176*/E178*/V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
 -E178*/G179*/V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;

-R176*/G177*/D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -G177*/E178*/D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -E178*/G179*/D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -R176*/G177*/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 5 -G177*/E178*/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -E178*/G179*/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -R176*/G177*/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -G177*/E178*/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -E178*/G179*/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 10 -N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
 -N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 15 /E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y
 /D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 20 -V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y
 /E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y
 /Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 25 /E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

30 Using SEQ ID NO: 6 as the backbone the following double
 mutantions resulting in the desired effect are contemplated
 according to the invention:

-R181*/G182*/N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -G182*/H183*/N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 35 -H183*/G184*/N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -R181*/G182*/I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V;
 -G182*/H183*/I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V;

-H183*/G184*/I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V;
 -R181*/G182*/E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -G182*/H183*/E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -H183*/G184*/E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 5 -R181*/G182*/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -G182*/H183*/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -H183*/G184*/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -R181*/G182*/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 -G182*/H183*/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 10 -H183*/G184*/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 -N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V;
 -N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 15 -N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 -I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V
 20 /E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V
 /E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V
 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 25 -E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 -E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 30 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

All Termamyl-like α -amylase defined above may suitably be used as backbone for preparing variants of the invention.

However, in a preferred embodiment the variant comprises the following mutations: N190F/Q264S in SEQ ID NO: 4 or in
 35 corresponding positions in another parent Termamyl-like α -amylases.

In another embodiment the variant of the invention comprises

the following mutations: I181*/G182*/N193F in SEQ ID NO: 3 (TVB146) or in corresponding positions in another parent Termamyl-like α -amylases. Said variant may further comprise a substitution in position E214Q.

5 In a preferred embodiment of the invention the parent Termamyl-like α -amylase is a hybrid α -amylase of SEQ ID NO: 4 and SEQ ID NO: 5. Specifically, the parent hybrid Termamyl-like α -amylase may be a hybrid alpha-amylase comprising the 445 C-terminal amino acid residues of the *B. licheniformis* α -amylase
10 shown in SEQ ID NO: 4 and the 37 N-terminal amino acid residues of the α -amylase derived from *B. amyloliquefaciens* shown in SEQ ID NO: 5, which may suitably further have the following mutations: H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4). The latter mentioned hybrid is used in the
15 examples below and is referred to as LE174.

General mutations in variants of the invention

It may be preferred that a variant of the invention comprises one or more modifications in addition to those outlined above.
20 Thus, it may be advantageous that one or more proline residues present in the part of the α -amylase variant which is modified is/are replaced with a non-proline residue which may be any of the possible, naturally occurring non-proline residues, and which preferably is an alanine, glycine, serine, threonine,
25 valine or leucine.

Analogously, it may be preferred that one or more cysteine residues present among the amino acid residues with which the parent α -amylase is modified is/are replaced with a non-cysteine residue such as serine, alanine, threonine, glycine, valine or
30 leucine.

Furthermore, a variant of the invention may - either as the only modification or in combination with any of the above outlined modifications - be modified so that one or more Asp and/or Glu present in an amino acid fragment corresponding to
35 the amino acid fragment 185-209 of SEQ ID NO: 4 is replaced by an Asn and/or Gln, respectively. Also of interest is the

replacement, in the Termamyl-like α -amylase, of one or more of the Lys residues present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID NO: 4 by an Arg.

It will be understood that the present invention encompasses
5 variants incorporating two or more of the above outlined modifications.

Furthermore, it may be advantageous to introduce point-mutations in any of the variants described herein.

10 Methods for preparing α -amylase variants

Several methods for introducing mutations into genes are known in the art. After a brief discussion of the cloning of α -amylase-encoding DNA sequences, methods for generating mutations at specific sites within the α -amylase-encoding sequence will be
15 discussed.

Cloning a DNA sequence encoding an α -amylase

The DNA sequence encoding a parent α -amylase may be isolated from any cell or microorganism producing the α -amylase in
20 question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the α -amylase to be studied. Then, if the amino acid sequence of the α -amylase is known, homologous, labelled oligonucleotide
25 probes may be synthesized and used to identify α -amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known α -amylase gene could be used as a probe to identify α -amylase-encoding clones,
30 using hybridization and washing conditions of lower stringency.

Yet another method for identifying α -amylase-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming α -amylase-negative bacteria with the resulting genomic DNA library, and

then plating the transformed bacteria onto agar containing a substrate for α -amylase, thereby allowing clones expressing the α -amylase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by S.L. Beaucage and M.H. Caruthers (1981) or the method described by Matthes et al. (1984). In the phosphoroamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988).

Site-directed mutagenesis

Once an α -amylase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the α -amylase-encoding sequence, is created in a vector carrying the α -amylase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984). US 4,760,025 discloses the introduction of oligonucleotides encoding multiple

mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

- 5 Another method for introducing mutations into α -amylase-encoding DNA sequences is described in Nelson and Long (1989). It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions.
- 10 From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Random Mutagenesis

15

Random mutagenesis is suitably performed either as localised or region-specific random mutagenesis in at least three parts of the gene translating to the amino acid sequence shown in question, or within the whole gene.

20

The random mutagenesis of a DNA sequence encoding a parent α -amylase may be conveniently performed by use of any method known in the art.

In relation to the above, a further aspect of the present invention relates to a method for generating a variant of a

25 parent α -amylase, e.g. wherein the variant exhibits altered or increased thermal stability relative to the parent, the method comprising:

- (a) subjecting a DNA sequence encoding the parent α -amylase to random mutagenesis,
- 30 (b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and
- (c) screening for host cells expressing an α -amylase variant which has an altered property (i.e. thermal stability) relative to the parent α -amylase.

Step (a) of the above method of the invention is preferably performed using doped primers.

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of
5 a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents. The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions,
10 and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) ir-radiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate
15 (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to
20 take place, and selecting for mutated DNA having the desired properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the
25 oligonucleotide at the positions which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the α -amylase enzyme by any published technique, using e.g. PCR, LCR or any DNA polymerase
30 and ligase as deemed appropriate.

Preferably, the doping is carried out using "constant random doping", in which the percentage of wild-type and mutation in each position is predefined. Furthermore, the doping may be directed toward a preference for the introduction of certain nucleotides, and thereby a preference for the introduction of one or more specific amino acid residues. The doping may be made, e.g., so as to allow for the introduction of 90% wild type and 10% mutations in each position. An additional consideration in the choice of a doping scheme is based on genetic as well as protein-structural constraints. The doping scheme may be made by using the DOPE program which, *inter alia*, ensures that introduction of stop codons is avoided.

When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent α -amylase is subjected to PCR under conditions that increase the mis-incorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol.1, 1989, pp. 11-15).

A mutator strain of *E. coli* (Fowler et al., Molec. Gen. Genet., 133, 1974, pp. 179-191), *S. cerevisiae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the α -amylase by, e.g., transforming a plasmid containing the parent glycosylase into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may be subsequently transformed into the expression organism.

The DNA sequence to be mutagenized may be conveniently present in a genomic or cDNA library prepared from an organism expressing the parent α -amylase. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or otherwise exposed to the mutagenising agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harboured in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to performing the expression step b) or the screening step c). Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenising agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are the following: gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans* or *Streptomyces murinus*; and gram-negative bacteria such as *E. coli*.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

Localized random mutagenesis

The random mutagenesis may be advantageously localized to a part of the parent α -amylase in question. This may, e.g., be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized, or region-specific, random mutagenesis is conveniently performed by use of PCR generated mutagenesis techniques as described above or any other suitable technique known in the art. Alternatively, the DNA sequence encoding the
5 part of the DNA sequence to be modified may be isolated, e.g., by insertion into a suitable vector, and said part may be subsequently subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

10 Alternative methods of providing α -amylase variants

Alternative methods for providing variants of the invention include gene shuffling method known in the art including the methods e.g. described in WO 95/22625 (from Affymax Technologies N.V.) and WO 96/00343 (from Novo Nordisk A/S).

15

Expression of α -amylase variants

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form,
20 using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence
25 encoding an α -amylase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which
30 exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the
35 host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected

to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of
5 suitable promoters for directing the transcription of the DNA sequence encoding an α -amylase variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis* α -amylase
10 gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α -amylase (*amyQ*), the promoters of the *Bacillus subtilis* *xylA* and *xylB* genes etc. For transcription in a fungal host, examples of useful promoters are those derived from
15 the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α -amylase, *A. niger* acid stable α -amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

20 The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α -amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same
25 sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

30 The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Fur-
35 thermore, the vector may comprise *Aspergillus* selection markers such as *amdS*, *argB*, *niaD* and *sC*, a marker giving rise to

hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In general, the *Bacillus* α -amylases mentioned herein comprise a preregion permitting secretion of the expressed protease into the culture medium. If desirable, this preregion may be replaced by a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention encoding an α -amylase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of an α -amylase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*,

Bacillus brevis, *Bacillus stearothermophilus*, *Bacillus alkalo-*
philus, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus*
circulans, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus*
thuringiensis, or *Streptomyces lividans* or *Streptomyces murinus*,
5 or gramnegative bacteria such as *E.coli*. The transformation of
the bacteria may, for instance, be effected by protoplast trans-
formation or by using competent cells in a manner known per se.

The yeast organism may favourably be selected from a species
of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces*
10 *cerevisiae*. The filamentous fungus may advantageously belong to
a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus*
niger. Fungal cells may be transformed by a process involving
protoplast formation and transformation of the protoplasts fol-
lowed by regeneration of the cell wall in a manner known per se.
15 A suitable procedure for transformation of *Aspergillus* host
cells is described in EP 238 023.

In yet a further aspect, the present invention relates to a
method of producing an α -amylase variant of the invention, which
method comprises cultivating a host cell as described above
20 under conditions conducive to the production of the variant and
recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any convention-
al medium suitable for growing the host cell in question and
obtaining expression of the α -amylase variant of the invention.
25 Suitable media are available from commercial suppliers or may be
prepared according to published recipes (e.g. as described in
catalogues of the American Type Culture Collection).

The α -amylase variant secreted from the host cells may con-
veniently be recovered from the culture medium by well-known
30 procedures, including separating the cells from the medium by
centrifugation or filtration, and precipitating proteinaceous
components of the medium by means of a salt such as ammonium
sulphate, followed by the use of chromatographic procedures such
as ion exchange chromatography, affinity chromatography, or the
35 like.

Industrial applications

The α -amylase variants of this invention possesses valuable properties allowing for a variety of industrial applications. In particular, enzyme variants of the invention are applicable as a component in washing, dishwashing and hard-surface cleaning
5 detergent compositions. Numerous variants are particularly useful in the production of sweeteners and ethanol from starch, and/or for textile desizing. Conditions for conventional starch-conversion processes, including starch liquefaction and/or saccharification processes, are described in, e.g., US 3,912,590
10 and in EP patent publications Nos. 252 730 and 63 909.

Production of sweeteners from starch:

A "traditional" process for conversion of starch to fructose syrups normally consists of three consecutive enzymatic
15 processes, viz. a liquefaction process followed by a saccharification process and an isomerization process. During the liquefaction process, starch is degraded to dextrans by an α -amylase (e.g. Termamyl™) at pH values between 5.5 and 6.2 and at temperatures of 95-160°C for a period of approx. 2 hours. In
20 order to ensure an optimal enzyme stability under these conditions, 1 mM of calcium is added (40 ppm free calcium ions).

After the liquefaction process the dextrans are converted into dextrose by addition of a glucoamylase (e.g. AMG™) and a debranching enzyme, such as an isoamylase or a pullulanase (e.g.
25 Promozyme™). Before this step the pH is reduced to a value below 4.5, maintaining the high temperature (above 95°C), and the liquefying α -amylase activity is denatured. The temperature is lowered to 60°C, and glucoamylase and debranching enzyme are added. The saccharification process proceeds for 24-72 hours.

30 After the saccharification process the pH is increased to a value in the range of 6-8, preferably pH 7.5, and the calcium is removed by ion exchange. The dextrose syrup is then converted into high fructose syrup using, e.g., an immobilized gluco-seisomerase (such as Sweetzyme™).

35 At least 1 enzymatic improvements of this process could be envisaged. Reduction of the calcium dependency of the liquefying α -amylase. Addition of free calcium is required to

ensure adequately high stability of the α -amylase, but free calcium strongly inhibits the activity of the glucoseisomerase and needs to be removed, by means of an expensive unit operation, to an extent which reduces the level of free calcium
5 to below 3-5 ppm. Cost savings could be obtained if such an operation could be avoided and the liquefaction process could be performed without addition of free calcium ions.

To achieve that, a less calcium-dependent Termamyl-like α -amylase which is stable and highly active at low
10 concentrations of free calcium (< 40 ppm) is required. Such a Termamyl-like α -amylase should have a pH optimum at a pH in the range of 4.5-6.5, preferably in the range of 4.5-5.5.

Detergent compositions

15 As mentioned above, variants of the invention may suitably be incorporated in detergent compositions. Increased thermostability at low calcium concentrations would be very beneficial for amylase performance in detergents, i.e. the alkaline region. Reference is made, for example, to WO 96/23874
20 and WO 97/07202 for further details concerning relevant ingredients of detergent compositions (such as laundry or dishwashing detergents), appropriate methods of formulating the variants in such detergent compositions, and for examples of relevant types of detergent compositions.

25 Detergent compositions comprising a variant of the invention may additionally comprise one or more other enzymes, such as a lipase, cutinase, protease, cellulase, peroxidase or laccase, and/or another α -amylase.

α -amylase variants of the invention may be incorporated in
30 detergents at conventionally employed concentrations. It is at present contemplated that a variant of the invention may be incorporated in an amount corresponding to 0.00001-1 mg (calculated as pure, active enzyme protein) of α -amylase per liter of wash/dishwash liquor using conventional dosing levels
35 of detergent.

The invention also relates to a composition comprising

a mixture of one or more variants of the invention derived from (as the parent Termamyl-like α -amylase) the *B. stearothermophilus* α -amylase having the sequence shown in SEQ ID NO: 3 and a Termamyl-like alpha-amylase derived from the *B. licheniformis* α -amylase having the sequence shown in SEQ ID NO: 4.

Further, the invention also relates to a composition comprising a mixture of one or more variants according the invention derived from (as the parent Termamyl-like α -amylase) the *B. stearothermophilus* α -amylase having the sequence shown in SEQ ID NO: 3 and a hybrid alpha-amylase comprising a part of the *B. amyloliquefaciens* α -amylase shown in SEQ ID NO: 5 and a part of the *B. licheniformis* α -amylase shown in SEQ ID NO: 4. The latter mentioned hydrid Termamyl-like α -amylase comprises the 445 C-terminal amino acid residues of the *B. licheniformis* α -amylase shown in SEQ ID NO: 4 and the 37 N-terminal amino acid residues of the α -amylase derived from *B. amyloliquefaciens* shown in SEQ ID NO: 5. Said latter mentioned hybrid α -amylase may suitably comprise the following mutations: H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4). In the examples below said hybrid parent Termamyl-like α -amylase, is used in combination with variants of the invention, which variants may be used in compositions of the invention.

In a specific embodiment of the invention the composition comprises a mixture of TVB146 and LE174, e.g., in a ratio of 2:1 to 1:2, such as 1:1.

A α -amylase variant of the invention or a composition of the invention may in an aspect of the invention be used for washing and/or dishwashing; for textile desizing or for starch liquefaction.

MATERIALS AND METHODS**Enzymes:**

BSG alpha-amylase: *B. stearothermophilus* alpha-amylase depicted
5 in SEQ ID NO: 3.

TVB146 alpha-amylase variant: *B. stearothermophilus* alpha-
amylase variant depicted in SEQ ID NO: 3 with the following
mutations: with the deletion in positions I181-G182 + N193F.

LE174 hybrid alpha-amylase variant:

10 LE174 is a hybrid Termamyl-like alpha-amylase being identical
to the Termamyl sequence, i.e., the *Bacillus licheniformis* α -
amylase shown in SEQ ID NO: 4, except that the N-terminal 35
amino acid residues (of the mature protein) has been replaced
by the N-terminal 33 residues of BAN (mature protein), i.e.,
15 the *Bacillus amyloliquefaciens* alpha-amylase shown in SEQ ID
NO: 5, which further have following mutations:

H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO:
4). LE174 was constructed by SOE-PCR (Higuchi et al. 1988,
Nucleic Acids Research 16:7351).

20

Fermentation and purification of α -amylase variants

A *B. subtilis* strain harbouring the relevant expression
plasmid is streaked on a LB-agar plate with 10 μ g/ml kanamycin
from -80°C stock, and grown overnight at 37°C.

25 The colonies are transferred to 100 ml BPX media supplemented
with 10 μ g/ml kanamycin in a 500 ml shaking flask.

Composition of BPX medium:

	Potato starch	100	g/l
	Barley flour	50	g/l
30	BAN 5000 SKB	0.1	g/l
	Sodium caseinate	10	g/l
	Soy Bean Meal	20	g/l
	Na ₂ HPO ₄ , 12 H ₂ O	9	g/l
	Pluronic™	0.1	g/l

35

The culture is shaken at 37°C at 270 rpm for 5 days.

Cells and cell debris are removed from the fermentation broth by centrifugation at 4500 rpm in 20-25 minutes. Afterwards the supernatant is filtered to obtain a completely clear solution.

5 The filtrate is concentrated and washed on a UF-filter (10000 cut off membrane) and the buffer is changed to 20mM Acetate pH 5.5. The UF-filtrate is applied on a S-sepharose F.F. and elution is carried out by step elution with 0.2M NaCl in the same buffer. The eluate is dialysed against 10mM Tris, pH 9.0 and applied on a

10 Q-sepharose F.F. and eluted with a linear gradient from 0-0.3M NaCl over 6 column volumes. The fractions which contain the activity (measured by the Phadebas assay) are pooled, pH was adjusted to pH 7.5 and remaining color was removed by a treatment with 0.5% W/vol. active coal in 5 minutes.

15

Activity determination - (KNU)

One Kilo alphah-amylase Unit (1 KNU) is the amount of enzyme which breaks down 5.26 g starch (Merck, Amylum Solubile, Erg. B 6,

20 Batch 9947275) per hour in Novo Nordisk's standard method for determination of alpha-amylase based upon the following condition:

Substrate	soluble starch
25 Calcium content in solvent	0.0043 M
Reaction time	7-20 minutes
Temperature	37°C
pH	5.6

30 Detailed description of Novo Nordisk's analytical method (AF 9) is available on request.

BS-amylase Activity Determination - KNU(S)**1. Application Field**

This method is used to determine α -amylase activity in fermentation and recovery samples and formulated and granulated products.

2. Principle

BS-amylase breaks down the substrate (4,6-ethylidene(G₇)-p-nitrophenyl(G₁)- α ,D-maltoheptaoside (written as ethylidene-G₇-PNP) into, among other things, G₂-PNP and G₃-PNP, where G denoted glucose and PNP p-nitrophenol.

G₂-PNP and G₃-PNP are broken down by α -glucosidase, which is added in excess, into glucose and the yellow-coloured p-nitrophenol.

The colour reaction is monitored in situ and the change in absorbance over time calculated as an expression of the speed of the reaction and thus of the activity of the enzyme. See the Boehringer Mannheim 1442 309 guidelines for further details.

2.1 Reaction conditions

Reaction:

Temperature : 37°C

pH : 7.1

Pre-incubation time: 2 minutes

Detection:

Wavelength : 405 nm

Measurement time 3 minutes

3. Definition of Units

Bacillus stearothermophilus alpha-amylase (BS-amylase) activity is determined relative to a standard of declared activity and stated in Kilo Novo Units (Stearothermophilus) or KNU(S)).

4. Specificity and Sensitivity

Limit of determination: approx. 0.4 KNU(s)/g

5. Apparatus

Cobas Fara analyser

Diluted (e.g. Hamilton Microlab 1000)

Analytical balance (e.g. Mettler AE 100)

5 Stirrer plates

6. Reagents/Substrates

A ready-made kit is used in this analysis to determine α -amylase activity. Note that the reagents specified for the substrate and

10 α -glucosidase are not used as described in the Boehringer Mannheim guidelines. However, the designations "buffer", "glass 1", glass 1a" and Glass 2" are those referred to in those guidelines.

15 6.1. Substrate

4,6-ethylidene(G_7)-p-nitrophenyl(G_1)- α ,D-maltoheptaoside (written as ethylidene- G_7 -PNP) e.g. Boehringer Mannheim 1442 309

6.2 α -glucosidase help reagent

20 α -glucosidase, e.g. Boehringer Mannheim 1442 309

6.3 BRIJ 35 solution

BRIJ 35 (30% W/V Sigma 430 AG-6)

1000 mL

Demineralized water

up to 2,000 mL

25

6.4 Stabiliser

Brij 35 solution

33 mL

 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Merck 2382)

882 g

Demineralized water

up to 2,000 mL

30

7. Samples and Standards7.1 Standard curve

35 Example: Preparation of BS-amylase standard curve

The relevant standard is diluted to 0.60 KNU(s)/mL as follows. A calculated quantity of standard is weighed out and added to 200 mL volumetric flask, which is filled to around the 2/3 mark with demineralized water. Stabiliser corresponding to 1% of the
 5 volume of the flask is added and the flask is filled to the mark with demineralized water.

A Hamilton Microlab 1000 is used to produce the dilutions shown below. Demineralized water with 1% stabiliser is used as the diluent.

10

Dilution No.	Enzyme stock solution	1% stabiliser	KNU(s)/mL
1	20µL	580µL	0.02
2	30µL	570µL	0.03
3	40µL	560µL	0.04
4	50µL	550µL	0.05
5	60µL	540µL	0.06

7.2 Level control

A Novo Nordisk A/S BS amylase level control is included in all runs using the Cobas Fara. The control is diluted with 1%
 15 stabiliser so that the final dilution is within the range of the standard curve. All weights and dilutions are noted on the worklist

7.3 Sample solutions

20 Single determination

Fermentation samples (not final samples) from production, all fermentation samples from pilot plants and storage stability samples are weighed out and analyzed once only.

Double determination over 1 run:

25 Process samples, final fermentation samples from production, samples from GLP studies and R&D samples are weighed out and analyzed twice.

Double determinations over 2 runs:

Finished product samples are weighed out and analyzed twice over two separate runs.

Maximum concentration of samples in powder form: 5%

5 Test samples are diluted with demineralized water with 1% stabiliser to approx. 0.037 KNU(S)/mL on the basis of their expected activity. The final dilution is made direct into the sample cup.

8. Procedure

10 8.1 Cobas Menu Program

- The Cobas Menu Program is used to suggest the weight/dilutions of samples and level control to be used.
- The samples are entered into the program with a unique identification code and a worklist is printed out
- 15 ■ The samples and control are weighed out and diluted as stated on the worklist with hand-written weight data is inserted into the BS-amylase analysis logbook
- The results are computered automatically by the Cobas Fara as described in item 9 and printed out along with the standard
- 20 curve.
- Worklists and results printouts are inserted into the BS-amylase analysis logbook.

8.2 Cobas Fara set-up

- 25 ■ The samples are placed in the sample rack
- The five standards are placed in the calibration rack at position 1 to 5 (strongest standard at position 5), and control placed in the same rack at position 10.
- The substrate is transferred to a 30 mL reagent container and
- 30 placed in that reagent rack at position 2 (holder 1).
- The α -glucosidase help reagent is transferred to a 50 mL reagent container and placed in the reagent rack at position 2 (holder C)

35 8.3 Cobas Fare analysis

The main principles of the analysis are as follows:

20µL sample and 10µL rinse-water are pipetted into the cuvette along with 250µL α-glucosidase help reagent. The cuvette rotates for 10 seconds and the reagents are thrown out into the horizontal cuvettes. 25µL substrate and 20µL rinse-water are pipetted off. After a 1 second wait to ensure that the temperature is 37°C, the cuvette rotates again and the substrate is mixed into the horizontal cuvettes. Absorbance is measured for the first time after 120 seconds and then every 5 seconds. Absorbance is measured a total of 37 times for each sample.

9. Calculations

The activity of the samples is calculated relative to Novo Nordisk A/S standard.

The standard curve is plotted by the analyzer. The curve is to be gently curved, rising steadily to an absorbance of around 0.25 for standard no. 5.

The activity of the samples in KNU(S)/mL is read off the standard curve by the analyzer.

The final calculations to allow for the weights/dilutions used employ the following formula:

$$\text{Activity in KNU(S)/g} = S \times V \times F/W$$

S= analysis result read off (KNU(S)/mL

V= volume of volumetric flask used in mL

F= dilution factor for second dilution

W= weight of enzyme sample in g

9.2 Calculation of mean values

Results are stated with 3 significant digits. However, for sample activity < 10 KNU(S)/g, only 2 significant digits are given.

The following rules apply on calculation of mean values:

1. Data which deviates more than 2 standard deviations from the mean value is not included in the calculation.

2. Single and double determination over one run:

The mean value is calculated on basis of results lying within the standard curve's activity area.

3. Double determinations over two runs: All values are included in the mean value. Outliers are omitted.

10. Accuracy and Precision

- 5 The coefficient of variation is 2.9% based on retrospective validation of analysis results for a number of finished products and the level control.

Assay for α -Amylase Activity

- 10 α -Amylase activity is determined by a method employing Phadebas® tablets as substrate. Phadebas tablets (Phadebas® Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-coloured starch polymer which has been mixed with bovine serum albumin and a buffer substance and
15 tabletted.

- For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM CaCl_2 , pH adjusted to the value of interest with NaOH). The test is
20 performed in a water bath at the temperature of interest. The α -amylase to be tested is diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this α -amylase solution is added to the 5 ml 50 mM Britton-Robinson buffer. The starch is hydrolysed by the α -amylase giving soluble blue fragments. The absorbance of
25 the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the α -amylase activity.

- It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range
30 there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion. Under a specified set of conditions (temp., pH, reaction time, buffer conditions) 1 mg of a given α -amylase will hydrolyse a certain amount of substrate and a blue colour
35 will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific

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activity (activity/mg of pure α -amylase protein) of the α -amylase in question under the given set of conditions.

EXAMPLES**EXAMPLE 1**Construction of variants of BSG α -amylase (SEQ ID NO: 3)

5 The gene encoding BSG, amyS, is located in plasmid pPL1117. This plasmid contains also the gene conferring resistance towards kanamycin and an origin of replication, both obtained from plasmid pUB110 (Gryczan, T.J. et al (1978) J.Bact 134:318-329).

10 The DNA sequence of the mature part of amyS is shown as SEQ ID NO: 11 and the amino acid sequence of the mature protein is shown as SEQ ID NO: 3

15 BSG variant TVB145, which contains a deletion of 6 nucleotides corresponding to amino acids I181-G182 in the mature protein, is constructed as follows:

 Polymerase Chain Reaction (PCR) is utilized to amplify the part of the amyS gene (from plasmid pPL1117), located between DNA primers BSG1 (SEQ ID NO: 15) and BSGM2 (SEQ ID NO: 18). BSG1 is identical to a part of the amyS gene whereas BSGM2
20 contains the 6 bp nucleotide deletion. A standard PCR reaction is carried out: 94°C for 5 minutes, 25 cycles of (94°C for 45 seconds, 50°C for 45 seconds, 72°C for 90 seconds), 72°C for 7 minutes using the Pwo polymerase under conditions as recommended by the manufacturer, Boehringer Mannheim GmbH.

25 The resulting approximately 550 bp amplified band was used as a megaprimer (Barik, S and Galinski, MS (1991): Biotechniques 10: 489-490) together with primer BSG3 in a second PCR with pPL1117 as template resulting in a DNA fragment of approximately 1080 bp.

30 This DNA fragment is digested with restriction endonucleases Acc65I and SalI and the resulting approximately 550 bp fragment is ligated into plasmid pPL1117 digested with the same enzymes and transformed into the protease- and amylase-deleted *Bacillus subtilis* strain SHA273 (described in
35 WO92/11357 and WO95/10603).

Kanamycin resistant and starch degrading transformants were analysed for the presence of the desired mutations (restriction

digest to verify the introduction of a HindIII site in the gene). The DNA sequence between restriction sites Acc65I and Sall was verified by DNA sequencing to ensure the presence of only the desired mutations.

5 BSG variant TVB146 which contains the same 6 nucleotide deletion as TVB145 and an additional substitution of asparagine 193 for a phenylalanine, N193F, was constructed in a similar way as TVB145 utilizing primer BSGM3 (SEQ ID NO: 19) in the first PCR.

10 BSG variant TVB161, containing the deletion of I181-G182, N193F, and L204F, is constructed in a similar way as the two previous variants except that the template for the PCR reactions is plasmid pTVB146 (pPL1117 containing the TVB146-mutations within amyS and the mutagenic oligonucleotide for the
15 first PCR is BSGM3.

BSG variant TVB162, containing the deletion of I181-G182, N193F, and E210H, is constructed in a similar way as TVB161 except that the mutagenic oligonucleotide is BSGM4 (SEQ ID NO: 20).

20 BSG variant TVB163, containing the deletion of I181-G182, N193F, and E214Q, is constructed in a similar way as TVB161 except that the mutagenic oligonucleotide is BSGM5 (SEQ ID NO: 21).

The above constructed BSG variants were then fermented and
25 purified as described above in the "Material and Methods" section.

EXAMPLE 2

Measurement of the calcium- and pH-dependent stability

30 Normally, the industrial liquefaction process runs using pH 6.0-6.2 as liquefaction pH and an addition of 40 ppm free calcium in order to improve the stability at 95°C-105°C. Some of the herein proposed substitutions have been made in order to improve the stability at

- 35 1. lower pH than pH 6.2 and/or
2. at free calcium levels lower than 40 ppm free calcium.

Two different methods have been used to measure the improvements in stability obtained by the different

substitutions in the α -amylase from *B.stearothermophilus*:

Method 1. One assay which measures the stability at reduced pH, pH 5.0, in the presence of 5 ppm free calcium.

10 μ g of the variant were incubated under the
5 following conditions: A 0.1 M acetate solution, pH adjusted to pH 5.0, containing 5ppm calcium and 5% w/w common corn starch (free of calcium). Incubation was made in a water bath at 95°C for 30 minutes.

Method 2. One assay which measure the stability in the
10 absence of free calcium and where the pH is maintained at pH 6.0. This assay measures the decrease in calcium sensitivity: 10 μ g of the variant were incubated under the following conditions: A 0.1 M acetate solution, pH adjusted to pH 6.0, containing 5% w/w common corn starch (free of
15 calcium). Incubation was made in a water bath at 95°C for 30 minutes.

Stability determination

All the stability trials 1, 2 have been made using
20 the same set up. The method was:

The enzyme was incubated under the relevant conditions (1-4). Samples were taken at 0, 5, 10, 15 and 30 minutes and diluted 25 times (same dilution for all taken samples) in assay buffer (0.1M 50mM Britton buffer pH 7.3) and the activity was
25 measured using the Phadebas assay (Pharmacia) under standard conditions pH 7.3, 37°C.

The activity measured before incubation (0 minutes) was used as reference (100%). The decline in percent was calculated as a function of the incubation time. The table shows the
30 residual activity after 30 minutes of incubation.

Stability method 1. / Low pH stability improvement

MINUTES OF INCUBATION	WT. SEQ. ID. NO:3 AMYLASE (BSG)	SEQ. ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 (TVB145)	SEQ. ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 + N193F (TVB146)	SEQ. ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 + N193F + E214Q (TVB163)
0	100	100	100	100
5	29	71	83	77
10	9	62	77	70
15	3	50	72	67
30	1	33	62	60

5 Stability method 1. / Low pH stability improvement

The temperature described in method 1 has been reduced from 95°C to 70°C since the amylases mentioned for SEQ ID NO: 1 and 2 have a lower thermostability than the one for SEQ ID NO: 3.

MINUTES OF INCUBATION	WT. SEQ. ID. NO: 2 AMYLASE	SEQ. ID NO: 2 VARIANT WITH DELETION IN POS. D183-G184	SEQ. ID NO: 1 AMYLASE	SEQ. ID NO: 1 VARIANT WITH DELETION IN POS. T183-G184
0	100	100	100	100
5	73	92	41	76
10	59	88	19	69
15	48	91	11	62
30	28	92	3	59

Stability method 2. / Low calcium sensitivity

MINUTES OF INCUBATION	WT. SEQ ID NO: 3 AMYLASE (BSG)	SEQ ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 (TVB145)	SEQ ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 + N193F (TVB146)	SEQ ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 + N193F + E214Q (TVB163)
0	100	100	100	100
5	60	82	81	82
10	42	76	80	83
15	31	77	81	79
30	15	67	78	79

Specific activity determination.

5 The specific activity was determined using the Phadebas assay (Pharmacia) as activity/mg enzyme. The activity was determined using the α -amylase assay described in the Materials and Methods section herein.

The specific activity of the parent enzyme and a single and
10 a double mutation was determined to:

BSG: SEQ ID NO:3 (Parent enzyme) 20000 NU/mg

TVB145: SEQ ID NO:3 with the deletion in positions
I181-G182: (Single mutation) 34600 NU/mg

15

TVB146: SEQ ID NO:3 with the deletion in positions
I181-G182 + N193F: (Double mutation)

36600 NU/mg

TVB163: SEQ ID NO:3 with the deletion in positions
20 I181-G182+N193F+E214Q: (Triple mutation) 36300 NU/mg

EXAMPLE 3

Pilot plant jet cook and liquefaction with alpha-amylase

variant TVB146

Pilot plant liquefaction experiments were run in the mini-jet system using a dosage of 50 NU (S)/g DS at pH 5.5 with 5 ppm added Ca^{++} , to compare the performance of formulated BSG alpha-amylase variant TVB146 (SEQ ID NO: 3 with deletion in positions

I181-G182 + N193F) with that of parent BSG alpha-amylase (SEQ ID NO: 3). The reaction was monitored by measuring the DE increase (Neocuproine method) as a function of time.

Corn starch slurries were prepared by suspending 11.8 kg Cerestar C*Pharm GL 03406 (89 % starch) in deionized water and making up to 30 kg. The pH was adjusted to 5.5 at ambient temperature, after the addition of 0.55 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.

The following enzymes were used:

15

TVB146	108 KNU(S)/g, 146 KNU(SM9)/g
BSG amylase	101 KNU(S)/g, 98 KNU(SM9)/g

An amount of enzyme corresponding to 50 NU (SM9)/g DS was added, and the conductivity adjusted to 300mS using NaCl. The standard conditions were as follows:

Substrate concentration	35 % w/w (initial) 31.6-31.9 % w/w (final)
25 Temperature	105°C, 5 min (Primary liquefaction) 95°C, 90 min (Secondary liquefaction)
pH (initial)	5.5

After jetting, the liquefied starch was collected and transported in sealed thermos-flasks from the pilot plant to the laboratory, where secondary liquefaction was continued at 95 °C.

10 ml samples were taken at 15 minute intervals from 15-90 minutes. 2 drops of 1 N HCl were added to inactivate the enzyme. From these samples, 0.3-0.1 g (according to the expected DE) were weighed out and diluted to 100 ml. Reducing sugars were then determined according to the Neocuproine method (Determination of reducing sugar with improved precision.

Dygert, Li, Florida and Thomas (1965). Anal. Biochem 13, 368) and DE values determined. The development of DE as a function of time is given in the following table:

	TVB146	BSG
Time (min.)	DE (neocuproine)	
15	2.80	2.32
30	4.88	3.56
45	6.58	4.98
60	8.17	6.00
75	9.91	7.40
90	11.23	8.03

As can be seen the alpha-amylase variant TVB146 performed significantly better under industrially relevant application conditions at low levels of calcium than the parent BSG alpha-amylase.

EXAMPLE 4

Jet Cook and Liquefaction with a combination of alpha-amylase variants (TVB146 and LE174)

Jet cook and liquefaction using a combination of the alpha-amylase variants, TVB146 and LE174 (ratio 1:1) were carried out at the following conditions:

Substrate A.E. Staley food grade powdered corn starch (100lbs)

D.S. 35% using DI water

Free Ca^{2+} 2.7ppm at pH 5.3 (none added, from the starch only)

Initial pH 5.3

Dose AF9 units (AF9 is available on request) for each enzyme variant was 28 NU/g starch db for a total dose of 56 NU/g

Temperature in primary liquefaction 105°C

Hold time in primary liquefaction 5 minutes

Temperature in secondary liquefaction 95°C

At 15 minutes into secondary liquefaction 1.5 gms of hydrolyzate was added to a tared one liter volumetric containing 500cc of DI water and 1 ml of one normal HCl and the exact wt. added was recorded. This was repeated at 15 minute intervals out to 90 minutes with an additional point at 127

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minutes. These were diluted to one liter and determined for dextrose equivalence via Neocuproine method as described by Dygert, Li, Florida and Thomas. Determination of reducing sugar with improved precision (1965). Anal. Biochem 13, 368.

5

The results were as follows:

	Time	DE
	15	3.2
	30	4.8
10	45	6.3
	60	7.8
	75	9.4
	90	10.4
	127	13.1

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1

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

5 (A) NAME: NOVO NORDISK A/S
 (B) STREET: Novo Alle
 (C) CITY: DK-2880 Bagsvaerd
 (E) COUNTRY: Denmark
 (F) POSTAL CODE (ZIP): DK-2880
 10 (G) TELEPHONE: +45 44 44 88 88
 (H) TELEFAX: +45 44 49 32 56

(ii) TITLE OF INVENTION: AMYLASE VARIANTS

(iii) NUMBER OF SEQUENCES: 21

(iv) COMPUTER READABLE FORM:

15 (A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

20 (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 485 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 25 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

30 His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
 1 5 10 15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala
 20 25 30

35 Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
 35 40 45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
 50 55 60

40 Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
 65 70 75 80

45 Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
 85 90 95

Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
 100 105 110

50 Gly Thr Glu Ile Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn
 115 120 125

Gln Glu Thr Ser Gly Glu Tyr Ala Ile Glu Ala Trp Thr Lys Phe Asp
 130 135 140

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	Phe	Pro	Gly	Arg	Gly	Asn	Asn	His	Ser	Ser	Phe	Lys	Trp	Arg	Trp	Tyr	145	150	155	160
5	His	Phe	Asp	Gly	Thr	Asp	Trp	Asp	Gln	Ser	Arg	Gln	Leu	Gln	Asn	Lys	165	170	175	
	Ile	Tyr	Lys	Phe	Arg	Gly	Thr	Gly	Lys	Ala	Trp	Asp	Trp	Glu	Val	Asp	180	185	190	
10	Thr	Glu	Asn	Gly	Asn	Tyr	Asp	Tyr	Leu	Met	Tyr	Ala	Asp	Val	Asp	Met	195	200	205	
	Asp	His	Pro	Glu	Val	Ile	His	Glu	Leu	Arg	Asn	Trp	Gly	Val	Trp	Tyr	210	215	220	
15	Thr	Asn	Thr	Leu	Asn	Leu	Asp	Gly	Phe	Arg	Ile	Asp	Ala	Val	Lys	His	225	230	235	240
	Ile	Lys	Tyr	Ser	Phe	Thr	Arg	Asp	Trp	Leu	Thr	His	Val	Arg	Asn	Thr	245	250	255	
20	Thr	Gly	Lys	Pro	Met	Phe	Ala	Val	Ala	Glu	Phe	Trp	Lys	Asn	Asp	Leu	260	265	270	
	Gly	Ala	Ile	Glu	Asn	Tyr	Leu	Asn	Lys	Thr	Ser	Trp	Asn	His	Ser	Val	275	280	285	
25	Phe	Asp	Val	Pro	Leu	His	Tyr	Asn	Leu	Tyr	Asn	Ala	Ser	Asn	Ser	Gly	290	295	300	
30	Gly	Tyr	Tyr	Asp	Met	Arg	Asn	Ile	Leu	Asn	Gly	Ser	Val	Val	Gln	Lys	305	310	315	320
	His	Pro	Thr	His	Ala	Val	Thr	Phe	Val	Asp	Asn	His	Asp	Ser	Gln	Pro	325	330	335	
35	Gly	Glu	Ala	Leu	Glu	Ser	Phe	Val	Gln	Gln	Trp	Phe	Lys	Pro	Leu	Ala	340	345	350	
	Tyr	Ala	Leu	Val	Leu	Thr	Arg	Glu	Gln	Gly	Tyr	Pro	Ser	Val	Phe	Tyr	355	360	365	
40	Gly	Asp	Tyr	Tyr	Gly	Ile	Pro	Thr	His	Gly	Val	Pro	Ala	Met	Lys	Ser	370	375	380	
45	Lys	Ile	Asp	Pro	Leu	Leu	Gln	Ala	Arg	Gln	Thr	Phe	Ala	Tyr	Gly	Thr	385	390	395	400
	Gln	His	Asp	Tyr	Phe	Asp	His	His	Asp	Ile	Ile	Gly	Trp	Thr	Arg	Glu	405	410	415	
50	Gly	Asn	Ser	Ser	His	Pro	Asn	Ser	Gly	Leu	Ala	Thr	Ile	Met	Ser	Asp	420	425	430	
55	Gly	Pro	Gly	Gly	Asn	Lys	Trp	Met	Tyr	Val	Gly	Lys	Asn	Lys	Ala	Gly				

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435

440

445

Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
 450 455 460

5

Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
 465 470 475 480

Val Trp Val Lys Gln
 485

10

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 485 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

15

20

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
 1 5 10 15

25

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ser
 20 25 30

Asn Leu Arg Asn Arg Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Trp
 35 40 45

30

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
 50 55 60

35

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
 65 70 75 80

Thr Arg Ser Gln Leu Glu Ser Ala Ile His Ala Leu Lys Asn Asn Gly
 85 90 95

40

Val Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
 100 105 110

Ala Thr Glu Asn Val Leu Ala Val Glu Val Asn Pro Asn Asn Arg Asn
 115 120 125

45

Gln Glu Ile Ser Gly Asp Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp
 130 135 140

Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp Arg Trp Tyr
 145 150 155 160

50

His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Gln Phe Gln Asn Arg
 165 170 175

55

Ile Tyr Lys Phe Arg Gly Asp Gly Lys Ala Trp Asp Trp Glu Val Asp
 180 185 190

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	Ser	Glu	Asn	Gly	Asn	Tyr	Asp	Tyr	Leu	Met	Tyr	Ala	Asp	Val	Asp	Met	
			195					200					205				
5	Asp	His	Pro	Glu	Val	Val	Asn	Glu	Leu	Arg	Arg	Trp	Gly	Glu	Trp	Tyr	
	210						215					220					
	Thr	Asn	Thr	Leu	Asn	Leu	Asp	Gly	Phe	Arg	Ile	Asp	Ala	Val	Lys	His	
10	225					230					235					240	
	Ile	Lys	Tyr	Ser	Phe	Thr	Arg	Asp	Trp	Leu	Thr	His	Val	Arg	Asn	Ala	
					245					250					255		
	Thr	Gly	Lys	Glu	Met	Phe	Ala	Val	Ala	Glu	Phe	Trp	Lys	Asn	Asp	Leu	
15				260					265					270			
	Gly	Ala	Leu	Glu	Asn	Tyr	Leu	Asn	Lys	Thr	Asn	Trp	Asn	His	Ser	Val	
		275						280					285				
20	Phe	Asp	Val	Pro	Leu	His	Tyr	Asn	Leu	Tyr	Asn	Ala	Ser	Asn	Ser	Gly	
	290						295					300					
	Gly	Asn	Tyr	Asp	Met	Ala	Lys	Leu	Leu	Asn	Gly	Thr	Val	Val	Gln	Lys	
25	305					310					315					320	
	His	Pro	Met	His	Ala	Val	Thr	Phe	Val	Asp	Asn	His	Asp	Ser	Gln	Pro	
					325					330					335		
30	Gly	Glu	Ser	Leu	Glu	Ser	Phe	Val	Gln	Glu	Trp	Phe	Lys	Pro	Leu	Ala	
				340					345					350			
	Tyr	Ala	Leu	Ile	Leu	Thr	Arg	Glu	Gln	Gly	Tyr	Pro	Ser	Val	Phe	Tyr	
		355						360					365				
35	Gly	Asp	Tyr	Tyr	Gly	Ile	Pro	Thr	His	Ser	Val	Pro	Ala	Met	Lys	Ala	
	370						375					380					
	Lys	Ile	Asp	Pro	Ile	Leu	Glu	Ala	Arg	Gln	Asn	Phe	Ala	Tyr	Gly	Thr	
40	385					390					395					400	
	Gln	His	Asp	Tyr	Phe	Asp	His	His	Asn	Ile	Ile	Gly	Trp	Thr	Arg	Glu	
					405					410					415		
	Gly	Asn	Thr	Thr	His	Pro	Asn	Ser	Gly	Leu	Ala	Thr	Ile	Met	Ser	Asp	
45				420					425					430			
	Gly	Pro	Gly	Gly	Glu	Lys	Trp	Met	Tyr	Val	Gly	Gln	Asn	Lys	Ala	Gly	
		435						440					445				
50	Gln	Val	Trp	His	Asp	Ile	Thr	Gly	Asn	Lys	Pro	Gly	Thr	Val	Thr	Ile	
	450						455					460					
	Asn	Ala	Asp	Gly	Trp	Ala	Asn	Phe	Ser	Val	Asn	Gly	Gly	Ser	Val	Ser	
55	465					470					475					480	

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Ile Trp Val Lys Arg
485

(2) INFORMATION FOR SEQ ID NO: 3:

- 5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 514 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 10 (ii) MOLECULE TYPE: peptide
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu
1 5 10 15
 Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala Asn Asn
20 25 30
 Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys
35 40 45
 Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
50 55 60
 Leu Gly Glu Phe Asn Gln Lys Gly Ala Val Arg Thr Lys Tyr Gly Thr
65 70 75 80
 Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
85 90 95
 Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
100 105 110
 Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
115 120 125
 Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
130 135 140
 Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145 150 155 160
 Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
165 170 175
 Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
180 185 190
 Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His
195 200 205
 Pro Glu Val Val Thr Glu Leu Lys Ser Trp Gly Lys Trp Tyr Val Asn
210 215 220
 Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys

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	225		230		235		240
	Phe Ser Phe Phe	Pro Asp Trp Leu Ser Asp Val Arg Ser Gln Thr Gly					
		245		250		255	
5	Lys Pro Leu Phe	Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys					
		260	265		270		
10	Leu His Asn Tyr	Ile Met Lys Thr Asn Gly Thr Met Ser Leu Phe Asp					
		275	280		285		
	Ala Pro Leu His	Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Thr					
		290	295		300		
15	Phe Asp Met Arg	Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro					
		305	310		315		320
	Thr Leu Ala Val	Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln					
		325	330			335	
20	Ala Leu Gln Ser	Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala					
		340	345			350	
	Phe Ile Leu Thr	Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp					
25		355	360		365		
	Tyr Tyr Gly Ile	Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile					
		370	375		380		
30	Asp Pro Leu Leu	Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His					
		385	390		395		400
	Asp Tyr Leu Asp	His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Val					
		405	410			415	
35	Thr Glu Lys Pro	Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro					
		420	425			430	
	Gly Gly Ser Lys	Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val					
40		435	440		445		
	Phe Tyr Asp Leu	Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser					
		450	455		460		
45	Asp Gly Trp Gly	Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp					
		465	470		475		480
	Val Pro Arg Lys	Thr Thr Val Ser Thr Ile Ala Trp Ser Ile Thr Thr					
		485	490			495	
50	Arg Pro Trp Thr	Asp Glu Phe Val Arg Trp Thr Glu Pro Arg Leu Val					
		500	505		510		
55	Ala Trp						

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 483 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro
1 5 10 15

Asn Asp Gly Gln His Trp Arg Arg Leu Gln Asn Asp Ser Ala Tyr Leu
20 25 30

Ala Glu His Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly
35 40 45

Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu
50 55 60

Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys
65 70 75 80

Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn
85 90 95

Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr
100 105 110

Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val
115 120 125

Ile Ser Gly Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro
130 135 140

Gly Arg Gly Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe
145 150 155 160

Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys
165 170 175

Phe Gln Gly Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn
180 185 190

Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val
195 200 205

Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln
210 215 220

Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe
225 230 235 240

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Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met
 245 250 255
 5 Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn
 260 265 270
 Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu
 275 280 285
 10 His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met
 290 295 300
 Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser
 305 310 315 320
 15 Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
 325 330 335
 Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
 340 345 350
 Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
 355 360 365
 25 Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile
 370 375 380
 Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His
 385 390 395 400
 30 Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp
 405 410 415
 Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
 420 425 430
 Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr
 435 440 445
 40 Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser
 450 455 460
 Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr
 465 470 475 480
 45 Val Gln Arg

(2) INFORMATION FOR SEQ ID NO: 5:

50

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 480 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

5	Val	Asn	Gly	Thr	Leu	Met	Gln	Tyr	Phe	Glu	Trp	Tyr	Thr	Pro	Asn	Asp	1	5	10	15
	Gly	Gln	His	Trp	Lys	Arg	Leu	Gln	Asn	Asp	Ala	Glu	His	Leu	Ser	Asp	20	25	30	
10	Ile	Gly	Ile	Thr	Ala	Val	Trp	Ile	Pro	Pro	Ala	Tyr	Lys	Gly	Leu	Ser	35	40	45	
	Gln	Ser	Asp	Asn	Gly	Tyr	Gly	Pro	Tyr	Asp	Leu	Tyr	Asp	Leu	Gly	Glu	50	55	60	
15	Phe	Gln	Gln	Lys	Gly	Thr	Val	Arg	Thr	Lys	Tyr	Gly	Thr	Lys	Ser	Glu	65	70	75	80
	Leu	Gln	Asp	Ala	Ile	Gly	Ser	Leu	His	Ser	Arg	Asn	Val	Gln	Val	Tyr	85	90	95	
20	Gly	Asp	Val	Val	Leu	Asn	His	Lys	Ala	Gly	Ala	Asp	Ala	Thr	Glu	Asp	100	105	110	
	Val	Thr	Ala	Val	Glu	Val	Asn	Pro	Ala	Asn	Arg	Asn	Gln	Glu	Thr	Ser	115	120	125	
25	Glu	Glu	Tyr	Gln	Ile	Lys	Ala	Trp	Thr	Asp	Phe	Arg	Phe	Pro	Gly	Arg	130	135	140	
	Gly	Asn	Thr	Tyr	Ser	Asp	Phe	Lys	Trp	His	Trp	Tyr	His	Phe	Asp	Gly	145	150	155	160
	Ala	Asp	Trp	Asp	Glu	Ser	Arg	Lys	Ile	Ser	Arg	Ile	Phe	Lys	Phe	Arg	165	170	175	
35	Gly	Glu	Gly	Lys	Ala	Trp	Asp	Trp	Glu	Val	Ser	Ser	Glu	Asn	Gly	Asn	180	185	190	
	Tyr	Asp	Tyr	Leu	Met	Tyr	Ala	Asp	Val	Asp	Tyr	Asp	His	Pro	Asp	Val	195	200	205	
40	Val	Ala	Glu	Thr	Lys	Lys	Trp	Gly	Ile	Trp	Tyr	Ala	Asn	Glu	Leu	Ser	210	215	220	
	Leu	Asp	Gly	Phe	Arg	Ile	Asp	Ala	Ala	Lys	His	Ile	Lys	Phe	Ser	Phe	225	230	235	240
	Leu	Arg	Asp	Trp	Val	Gln	Ala	Val	Arg	Gln	Ala	Thr	Gly	Lys	Glu	Met	245	250	255	
50	Phe	Thr	Val	Ala	Glu	Tyr	Trp	Gln	Asn	Asn	Ala	Gly	Lys	Leu	Glu	Asn	260	265	270	
	Tyr	Leu	Asn	Lys	Thr	Ser	Phe	Asn	Gln	Ser	Val	Phe	Asp	Val	Pro	Leu	275	280	285	

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His Phe Asn Leu Gln Ala Ala Ser Ser Gln Gly Gly Gly Tyr Asp Met
 290 295 300

5 Arg Arg Leu Leu Asp Gly Thr Val Val Ser Arg His Pro Glu Lys Ala
 305 310 315 320

Val Thr Phe Val Glu Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
 325 330 335

10 Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
 340 345 350

15 Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
 355 360 365

Thr Lys Gly Thr Ser Pro Lys Glu Ile Pro Ser Leu Lys Asp Asn Ile
 370 375 380

20 Glu Pro Ile Leu Lys Ala Arg Lys Glu Tyr Ala Tyr Gly Pro Gln His
 385 390 395 400

Asp Tyr Ile Asp His Pro Asp Val Ile Gly Trp Thr Arg Glu Gly Asp
 405 410 415

25 Ser Ser Ala Ala Lys Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
 420 425 430

Gly Gly Ser Lys Arg Met Tyr Ala Gly Leu Lys Asn Ala Gly Glu Thr
 435 440 445

Trp Tyr Asp Ile Thr Gly Asn Arg Ser Asp Thr Val Lys Ile Gly Ser
 450 455 460

35 Asp Gly Trp Gly Glu Phe His Val Asn Asp Gly Ser Val Ser Ile Tyr
 465 470 475 480

- 40 (2) INFORMATION FOR SEQ ID NO: 6:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 485 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- 45 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
 1 5 10 15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Asn Ser Asp Ala Ser
 20 25 30

55

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	Asn	Leu	Lys	Ser	Lys	Gly	Ile	Thr	Ala	Val	Trp	Ile	Pro	Pro	Ala	Trp	
			35					40					45				
5	Lys	Gly	Ala	Ser	Gln	Asn	Asp	Val	Gly	Tyr	Gly	Ala	Tyr	Asp	Leu	Tyr	
		50					55					60					
	Asp	Leu	Gly	Glu	Phe	Asn	Gln	Lys	Gly	Thr	Val	Arg	Thr	Lys	Tyr	Gly	
	65					70					75					80	
10	Thr	Arg	Ser	Gln	Leu	Gln	Ala	Ala	Val	Thr	Ser	Leu	Lys	Asn	Asn	Gly	
					85					90					95		
	Ile	Gln	Val	Tyr	Gly	Asp	Val	Val	Met	Asn	His	Lys	Gly	Gly	Ala	Asp	
15				100					105					110			
	Ala	Thr	Glu	Met	Val	Arg	Ala	Val	Glu	Val	Asn	Pro	Asn	Asn	Arg	Asn	
			115					120					125				
20	Gln	Glu	Val	Thr	Gly	Glu	Tyr	Thr	Ile	Glu	Ala	Trp	Thr	Arg	Phe	Asp	
		130					135					140					
	Phe	Pro	Gly	Arg	Gly	Asn	Thr	His	Ser	Ser	Phe	Lys	Trp	Arg	Trp	Tyr	
	145					150					155					160	
25	His	Phe	Asp	Gly	Val	Asp	Trp	Asp	Gln	Ser	Arg	Arg	Leu	Asn	Asn	Arg	
					165					170					175		
	Ile	Tyr	Lys	Phe	Arg	Gly	His	Gly	Lys	Ala	Trp	Asp	Trp	Glu	Val	Asp	
30				180					185					190			
	Thr	Glu	Asn	Gly	Asn	Tyr	Asp	Tyr	Leu	Met	Tyr	Ala	Asp	Ile	Asp	Met	
			195					200					205				
35	Asp	His	Pro	Glu	Val	Val	Asn	Glu	Leu	Arg	Asn	Trp	Gly	Val	Trp	Tyr	
		210					215					220					
	Thr	Asn	Thr	Leu	Gly	Leu	Asp	Gly	Phe	Arg	Ile	Asp	Ala	Val	Lys	His	
	225					230					235					240	
40	Ile	Lys	Tyr	Ser	Phe	Thr	Arg	Asp	Trp	Ile	Asn	His	Val	Arg	Ser	Ala	
					245					250					255		
	Thr	Gly	Lys	Asn	Met	Phe	Ala	Val	Ala	Glu	Phe	Trp	Lys	Asn	Asp	Leu	
45				260					265					270			
	Gly	Ala	Ile	Glu	Asn	Tyr	Leu	Gln	Lys	Thr	Asn	Trp	Asn	His	Ser	Val	
		275						280					285				
50	Phe	Asp	Val	Pro	Leu	His	Tyr	Asn	Leu	Tyr	Asn	Ala	Ser	Lys	Ser	Gly	
		290					295					300					
	Gly	Asn	Tyr	Asp	Met	Arg	Asn	Ile	Phe	Asn	Gly	Thr	Val	Val	Gln	Arg	
	305					310					315					320	
55	His	Pro	Ser	His	Ala	Val	Thr	Phe	Val	Asp	Asn	His	Asp	Ser	Gln	Pro	

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	325	330	335
5	Glu Glu Ala Leu Glu Ser Phe Val 340	Glu Glu Trp Phe Lys 345	Pro Leu Ala 350
	Tyr Ala Leu Thr Leu Thr Arg 355	Glu Gln Gly Tyr Pro 360	Ser Val Phe Tyr 365
10	Gly Asp Tyr Tyr Gly Ile Pro Thr 370	His Gly Val Pro Ala Met Arg Ser 375	
	Lys Ile Asp Pro Ile Leu Glu Ala Arg 385	Gln Lys Tyr Ala Tyr Gly Lys 390	
15	Gln Asn Asp Tyr Leu Asp His His 405	Asn Ile Ile Gly Trp Thr Arg Glu 410	
	Gly Asn Thr Ala His Pro Asn Ser 420	Gly Leu Ala Thr Ile Met Ser Asp 425	
20	Gly Ala Gly Gly Ser Lys Trp Met Phe 435	Val Gly Arg Asn Lys Ala Gly 440	
	Gln Val Trp Ser Asp Ile Thr Gly Asn Arg 450	Thr Gly Thr Val Thr Ile 455	
25	Asn Ala Asp Gly Trp Gly Asn Phe Ser Val 465	Asn Gly Gly Ser Val Ser 470	
	Ile Trp Val Asn Lys 485		

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
- 35 (A) LENGTH: 485 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

- 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

1	His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr	15
5		
10		
45	Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala	30
20		
25		
35	Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp	45
40		
50	Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr	60
55		
65	Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly	80
70		
75		

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	Thr	Arg	Asn	Gln	Leu	Gln	Ala	Ala	Val	Thr	Ser	Leu	Lys	Asn	Asn	Gly	
					85					90					95		
5	Ile	Gln	Val	Tyr	Gly	Asp	Val	Val	Met	Asn	His	Lys	Gly	Gly	Ala	Asp	
				100					105					110			
	Gly	Thr	Glu	Ile	Val	Asn	Ala	Val	Glu	Val	Asn	Arg	Ser	Asn	Arg	Asn	
			115					120					125				
10	Gln	Glu	Thr	Ser	Gly	Glu	Tyr	Ala	Ile	Glu	Ala	Trp	Thr	Lys	Phe	Asp	
		130					135					140					
	Phe	Pro	Gly	Arg	Gly	Asn	Asn	His	Ser	Ser	Phe	Lys	Trp	Arg	Trp	Tyr	
15	145					150					155					160	
	His	Phe	Asp	Gly	Thr	Asp	Trp	Asp	Gln	Ser	Arg	Gln	Leu	Gln	Asn	Lys	
				165						170					175		
20	Ile	Tyr	Lys	Phe	Arg	Gly	Thr	Gly	Lys	Ala	Trp	Asp	Trp	Glu	Val	Asp	
				180					185					190			
	Thr	Glu	Asn	Gly	Asn	Tyr	Asp	Tyr	Leu	Met	Tyr	Ala	Asp	Val	Asp	Met	
25			195					200					205				
	Asp	His	Pro	Glu	Val	Ile	His	Glu	Leu	Arg	Asn	Trp	Gly	Val	Trp	Tyr	
		210					215					220					
30	Thr	Asn	Thr	Leu	Asn	Leu	Asp	Gly	Phe	Arg	Ile	Asp	Ala	Val	Lys	His	
	225					230					235					240	
	Ile	Lys	Tyr	Ser	Phe	Thr	Arg	Asp	Trp	Leu	Thr	His	Val	Arg	Asn	Thr	
				245						250					255		
35	Thr	Gly	Lys	Pro	Met	Phe	Ala	Val	Ala	Glu	Phe	Trp	Lys	Asn	Asp	Leu	
				260					265					270			
	Gly	Ala	Ile	Glu	Asn	Tyr	Leu	Asn	Lys	Thr	Ser	Trp	Asn	His	Ser	Val	
40			275					280					285				
	Phe	Asp	Val	Pro	Leu	His	Tyr	Asn	Leu	Tyr	Asn	Ala	Ser	Asn	Ser	Gly	
		290					295					300					
45	Gly	Tyr	Tyr	Asp	Met	Arg	Asn	Ile	Leu	Asn	Gly	Ser	Val	Val	Gln	Lys	
	305					310					315					320	
	His	Pro	Thr	His	Ala	Val	Thr	Phe	Val	Asp	Asn	His	Asp	Ser	Gln	Pro	
				325						330					335		
50	Gly	Glu	Ala	Leu	Glu	Ser	Phe	Val	Gln	Gln	Trp	Phe	Lys	Pro	Leu	Ala	
				340					345					350			
	Tyr	Ala	Leu	Val	Leu	Thr	Arg	Glu	Gln	Gly	Tyr	Pro	Ser	Val	Phe	Tyr	
55			355					360					365				

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Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
 370 375 380

5 Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Phe Ala Tyr Gly Thr
 385 390 395 400

Gln His Asp Tyr Phe Asp His His Asp Ile Ile Gly Trp Thr Arg Glu
 405 410 415

10 Gly Asn Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
 420 425 430

Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys Asn Lys Ala Gly
 435 440 445

15 Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
 450 455 460

20 Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
 465 470 475 480

Val Trp Val Lys Gln
 485

25 (2) INFORMATION FOR SEQ ID NO: 8:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 485 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 30 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: peptide
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

35 His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
 1 5 10 15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ser
 20 25 30

40 Asn Leu Arg Asn Arg Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Trp
 35 40 45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
 50 55 60

45 Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
 65 70 75 80

50 Thr Arg Ser Gln Leu Glu Ser Ala Ile His Ala Leu Lys Asn Asn Gly
 85 90 95

Val Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
 100 105 110

55 Ala Thr Glu Asn Val Leu Ala Val Glu Val Asn Pro Asn Asn Arg Asn

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	115	120	125
5	Gln Glu Ile Ser Gly Asp Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp 130 135 140		
	Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp Arg Trp Tyr 145 150 155 160		
10	His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Gln Phe Gln Asn Arg 165 170 175		
	Ile Tyr Lys Phe Arg Gly Asp Gly Lys Ala Trp Asp Trp Glu Val Asp 180 185 190		
15	Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met 195 200 205		
	Asp His Pro Glu Val Val Asn Glu Leu Arg Arg Trp Gly Glu Trp Tyr 210 215 220		
20	Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His 225 230 235 240		
	Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Ala 245 250 255		
25	Thr Gly Lys Glu Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu 260 265 270		
	Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val 275 280 285		
30	Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly 290 295 300		
	Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys 305 310 315 320		
35	His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro 325 330 335		
	Gly Glu Ser Leu Glu Ser Phe Val Gln Glu Trp Phe Lys Pro Leu Ala 340 345 350		
40	Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr 355 360 365		
	Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala 370 375 380		
45	Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Asn Phe Ala Tyr Gly Thr 385 390 395 400		
	Gln His Asp Tyr Phe Asp His His Asn Ile Ile Gly Trp Thr Arg Glu 405 410 415		
50			
55			

	CATCATAATG	GAACAAATGG	TACTATGATG	CAATATTTTCG	AATGGTATTT	GCCAAATGAC	60
	GGAATCATT	GGAACAGGTT	GAGGGATGAC	GCAGCTAACT	TAAAGAGTAA	AGGGATAACA	120
30	GCTGTATGGA	TCCCACCTGC	ATGGAAGGGG	ACTTCCCAGA	ATGATGTAGG	TTATGGAGCC	180
	TATGATTTAT	ATGATCTTGG	AGAGTTTAAC	CAGAAGGGGA	CGGTTTCGTAC	AAAATATGGA	240
	ACACGCAACC	AGCTACAGGC	TGCGGTGACC	TCTTTAAAAA	ATAACGGCAT	TCAGGTATAT	300
35	GGTGATGTCG	TCATGAATCA	TAAAGGTGGA	GCAGATGGTA	CGGAAATTGT	AAATGCGGTA	360
	GAAGTGAATC	GGAGCAACCG	AAACCAGGAA	ACCTCAGGAG	AGTATGCAAT	AGAAGCGTGG	420
40	ACAAAGTTTG	ATTTTCCTGG	AAGAGGAAAT	AACCATTCCA	GCTTTAAGTG	GCGCTGGTAT	480
	CATTTTGATG	GGACAGATTG	GGATCAGTCA	CGCCAGCTTC	AAAACAAAAT	ATATAAATTC	540
	AGGGGAACAG	GCAAGGCCTG	GGACTGGGAA	GTCGATACAG	AGAATGGCAA	CTATGACTAT	600
45	CTTATGTATG	CAGACGTGGA	TATGGATCAC	CCAGAAGTAA	TACATGAACT	TAGAAACTGG	660
	GGAGTGTGGT	ATACGAATAC	ACTGAACCTT	GATGGATTTA	GAATAGATGC	AGTGAAACAT	720
50	ATAAAATATA	GCTTTACGAG	AGATTGGCTT	ACACATGTGC	GTAACACCAC	AGGTAAACCA	780
	ATGTTTGCAG	TGGCTGAGTT	TTGGAAAAAT	GACCTTGGTG	CAATTGAAAA	CTATTTGAAT	840
55	AAAACAAGTT	GGAATCACTC	GGTGTTTGAT	GTCCTCTCC	ACTATAATTT	GTACAATGCA	900

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TCTAATAGCG GTGGTTATTA TGATATGAGA AATATTTTAA ATGGTTCTGT GGTGCAAAAA 960
 CATCCAACAC ATGCCGTTAC TTTTGTTGAT AACCATGATT CTCAGCCCGG GGAAGCATTG 1020
 5 GAATCCTTTG TTCAACAATG GTTTAAACCA CTTGCATATG CATTGGTTCT GACAAGGGAA 1080
 CAAGGTTATC CTTCCGTATT TTATGGGGAT TACTACGGTA TCCCAACCCA TGGTGTTCGG 1140
 GCTATGAAAT CTAAAATAGA CCCTCTTCTG CAGGCACGTC AAACTTTTCG CTATGGTACG 1200
 10 CAGCATGATT ACTTTGATCA TCATGATATT ATCGGTTGGA CAAGAGAGGG AAATAGCTCC 1260
 CATCCAAATT CAGGCCTTGC CACCATTATG TCAGATGGTC CAGGTGGTAA CAAATGGATG 1320
 15 TATGTGGGGA AAAATAAAGC GGGACAAGTT TGGAGAGATA TTACCGGAAA TAGGACAGGC 1380
 ACCGTCACAA TTAATGCAGA CGGATGGGGT AATTCTCTG TTAATGGAGG GTCCGTTTCG 1440
 GTTTGGGTGA AGCAA 1455
 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1455 base pairs

(B) TYPE: nucleic acid

25 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

30 CATCATAATG GGACAAATGG GACGATGATG CAATACTTTG AATGGCACTT GCCTAATGAT 60
 GGGAATCACT GGAATAGATT AAGAGATGAT GCTAGTAATC TAAGAAATAG AGGTATAACC 120
 GCTATTTGGA TTCCGCCTGC CTGGAAAGGG ACTTCGCAA ATGATGTGGG GTATGGAGCC 180
 35 TATGATCTTT ATGATTTAGG GGAATTTAAT CAAAAGGGGA CGGTTCGTAC TAAGTATGGG 240
 ACACGTAGTC AATTGGAGTC TGCCATCCAT GCTTTAAAGA ATAATGGCGT TCAAGTTTAT 300
 40 GGGGATGTAG TGATGAACCA TAAAGGAGGA GCTGATGCTA CAGAAAACGT TCTTGCTGTC 360
 GAGGTGAATC CAAATAACCG GAATCAAGAA ATATCTGGGG ACTACACAAT TGAGGCTTGG 420
 ACTAAGTTTG ATTTTCCAGG GAGGGGTAAT ACATACTCAG ACTTTAAATG GCGTTGGTAT 480
 45 CATTTGATG GTGTAGATTG GGATCAATCA CGACAATTCC AAAATCGTAT CTACAAATTC 540
 CGAGGTGATG GTAAGGCATG GGATTGGGAA GTAGATTCGG AAAATGGAAA TTATGATTAT 600
 50 TTAATGTATG CAGATGTAGA TATGGATCAT CCGGAGGTAG TAAATGAGCT TAGAAGATGG 660
 GGAGAATGGT ATACAAATAC ATTAAATCTT GATGGATTTA GGATCGATGC GGTGAAGCAT 720
 ATTAAATATA GCTTTACACG TGATTGGTTG ACCCATGTAA GAAACGCAAC GGGAAAAGAA 780
 55

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	ATGTTTGCTG TTGCTGAATT TTGGAAAAAT GATTTAGGTG CCTTGGAGAA CTATTTAAAT	840
	AAAACAAACT GGAATCATTG TGTCTTTGAT GTCCCCCTTC ATTATAATCT TTATAACGCG	900
5	TCAAATAGTG GAGGCAACTA TGACATGGCA AAACCTCTTA ATGGAACGGT TGTTCAAAG	960
	CATCCAATGC ATGCCGTAAC TTTTGTGGAT AATCACGATT CTCAACCTGG GGAATCATTA	1020
10	GAATCATTTG TACAAGAATG GTTTAAGCCA CTTGCTTATG CGCTTATTTT AACAGAGAA	1080
	CAAGGCTATC CCTCTGTCTT CTATGGTGAC TACTATGGAA TTCCAACACA TAGTGTCCCA	1140
	GCAATGAAAG CCAAGATTGA TCCAATCTTA GAGGCGCGTC AAAATTTTGC ATATGGAACA	1200
15	CAACATGATT ATTTTGACCA TCATAATATA ATCGGATGGA CACGTGAAGG AAATACCACG	1260
	CATCCCAATT CAGGACTTGC GACTATCATG TCGGATGGGC CAGGGGGAGA GAAATGGATG	1320
20	TACGTAGGGC AAAATAAAGC AGGTCAAGTT TGGCATGACA TAACTGGAAA TAAACCAGGA	1380
	ACAGTTACGA TCAATGCAGA TGGATGGGCT AATTTTTCAG TAAATGGAGG ATCTGTTTCC	1440
	ATTTGGGTGA AACGA	1455
25	(2) INFORMATION FOR SEQ ID NO: 11:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1548 base pairs	
	(B) TYPE: nucleic acid	
30	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: DNA (genomic)	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:	
35	GCCGCACCGT TTAACGGCAC CATGATGCAG TATTTTGAAT GGTACTTGCC GGATGATGGC	60
	ACGTTATGGA CCAAAGTGGC CAATGAAGCC AACAACTTAT CCAGCCTTGG CATCACCGCT	120
40	CTTTGGCTGC CGCCCGCTTA CAAAGGAACA AGCCGCAGCG ACGTAGGGTA CGGAGTATAC	180
	GACTTGTATG ACCTCGGCGA ATTCAATCAA AAAGGGACCG TCCGCACAAA ATACGGAACA	240
	AAAGCTCAAT ATCTTCAAGC CATTCAAGCC GCCCAGCCG CTGGAATGCA AGTGACGCC	300
45	GATGTCGTGT TCGACCATAA AGGCGGCGCT GACGGCACGG AATGGGTGGA CGCCGTCGAA	360
	GTCAATCCGT CCGACCGCAA CCAAGAAATC TCGGGCACCT ATCAAATCCA AGCATGGACG	420
50	AAATTTGATT TTCCCGGGCG GGGCAACACC TACTCCAGCT TTAAGTGGCG CTGGTACCAT	480
	TTTGACGGCG TTGATTGGGA CGAAAGCCGA AAATTGAGCC GCATTTACAA ATTCCGCGGC	540
	ATCGGCAAAG CGTGGGATTG GGAAGTAGAC ACGGAAAACG GAAACTATGA CTACTTAATG	600
55	TATGCCGACC TTGATATGGA TCATCCCGAA GTCGTGACCG AGCTGAAAAA CTGGGGGAAA	660

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TGGTATGTCA ACACAACGAA CATTGATGGG TTCCGGCTTG ATGCCGTCAA GCATATTAAG 720
 TTCAGTTTTT TTCCTGATTG GTTGTCGTAT GTGCGTTCTC AGACTGGCAA GCCGCTATTT 780
 5 ACCGTCGGGG AATATTGGAG CTATGACATC AACAAGTTGC ACAATTACAT TACGAAAACA 840
 GACGGAACGA TGTCTTTGTT TGATGCCCCG TTACACAACA AATTTTATAC CGCTTCCAAA 900
 10 TCAGGGGGCG CATTGATAT GCGCACGTTA ATGACCAATA CTCTCATGAA AGATCAACCG 960
 ACATTGGCCG TCACCTTCGT TGATAATCAT GACACCGAAC CCGGCCAAGC GCTGCAGTCA 1020
 TGGGTCGACC CATGGTTCAA ACCGTTGGCT TACGCCTTTA TTCTAACTCG GCAGGAAGGA 1080
 15 TACCCGTGCG TCTTTTATGG TGACTATTAT GGCATTCCAC AATATAACAT TCCTTCGCTG 1140
 AAAAGCAAAA TCGATCCGCT CCTCATCGCG CGCAGGGATT ATGCTTACGG AACGCAACAT 1200
 20 GATTATCTTG ATCACTCCGA CATCATCGGG TGGACAAGGG AAGGGGGCAC TGAAAAACCA 1260
 GGATCCGGAC TGGCCGCACT GATCACCGAT GGGCCGGGAG GAAGCAAATG GATGTACGTT 1320
 GGCAAACAAC ACGCTGGAAA AGTGTTCTAT GACCTTACCG GCAACCGGAG TGACACCGTC 1380
 25 ACCATCAACA GTGATGGATG GGGGGAATTC AAAGTCAATG GCGGTTCGGT TTCGGTTTGG 1440
 GTTCCTAGAA AAACGACCGT TTCTACCATC GCTCGGCCGA TCACAACCCG ACCGTGGACT 1500
 30 GGTGAATTCG TCCGTTGGAC CGAACCACGG TTGGTGGCAT GGCCTTGA 1548

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
 35 (A) LENGTH: 1920 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

40 (ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 421..1872

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

45 CGGAAGATTG GAAGTACAAA AATAAGCAAA AGATTGTCAA TCATGTCATG AGCCATGCGG 60
 GAGACGGAAA AATCGTCTTA ATGCACGATA TTTATGCAAC GTTCGCAGAT GCTGCTGAAG 120
 AGATTATTAA AAAGCTGAAA GCAAAGGCT ATCAATTGGT AACTGTATCT CAGCTTGAAG 180
 50 AAGTGAAGAA GCAGAGAGGC TATTGAATAA ATGAGTAGAA GCGCCATATC GGCGCTTTTC 240
 TTTTGGAAGA AAATATAGGG AAAATGGTAC TTGTTAAAAA TTCGGAATAT TTATAACA 300
 55 TCATATGTTT CACATTGAAA GGGGAGGAGA ATCATGAAAC AACAAAACG GCTTTACGCC 360

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	CGATTGCTGA CGCTGTTATT TGCGCTCATC TTCTTGCTGC CTCATTCTGC AGCAGCGGCG	420
5	GCA AAT CTT AAT GGG ACG CTG ATG CAG TAT TTT GAA TGG TAC ATG CCC	468
	AAT GAC GGC CAA CAT TGG AGG CGT TTG CAA AAC GAC TCG GCA TAT TTG	516
	GCT GAA CAC GGT ATT ACT GCC GTC TGG ATT CCC CCG GCA TAT AAG GGA	564
10	ACG AGC CAA GCG GAT GTG GGC TAC GGT GCT TAC GAC CTT TAT GAT TTA	612
	GGG GAG TTT CAT CAA AAA GGG ACG GTT CGG ACA AAG TAC GGC ACA AAA	660
15	GGA GAG CTG CAA TCT GCG ATC AAA AGT CTT CAT TCC CGC GAC ATT AAC	708
	GTT TAC GGG GAT GTG GTC ATC AAC CAC AAA GGC GGC GCT GAT GCG ACC	756
	GAA GAT GTA ACC GCG GTT GAA GTC GAT CCC GCT GAC CGC AAC CGC GTA	804
20	ATT TCA GGA GAA CAC CTA ATT AAA GCC TGG ACA CAT TTT CAT TTT CCG	852
	GGG CGC GGC AGC ACA TAC AGC GAT TTT AAA TGG CAT TGG TAC CAT TTT	900
25	GAC GGA ACC GAT TGG GAC GAG TCC CGA AAG CTG AAC CGC ATC TAT AAG	948
	TTT CAA GGA AAG GCT TGG GAT TGG GAA GTT TCC AAT GAA AAC GGC AAC	996
	TAT GAT TAT TTG ATG TAT GCC GAC ATC GAT TAT GAC CAT CCT GAT GTC	1044
30	GCA GCA GAA ATT AAG AGA TGG GGC ACT TGG TAT GCC AAT GAA CTG CAA	1092
	TTG GAC GGT TTC CGT CTT GAT GCT GTC AAA CAC ATT AAA TTT TCT TTT	1140
35	TTG CGG GAT TGG GTT AAT CAT GTC AGG GAA AAA ACG GGG AAG GAA ATG	1188
	TTT ACG GTA GCT GAA TAT TGG CAG AAT GAC TTG GGC GCG CTG GAA AAC	1236
	TAT TTG AAC AAA ACA AAT TTT AAT CAT TCA GTG TTT GAC GTG CCG CTT	1284
40	CAT TAT CAG TTC CAT GCT GCA TCG ACA CAG GGA GGC GGC TAT GAT ATG	1332
	AGG AAA TTG CTG AAC GGT ACG GTC GTT TCC AAG CAT CCG TTG AAA TCG	1380
45	GTT ACA TTT GTC GAT AAC CAT GAT ACA CAG CCG GGG CAA TCG CTT GAG	1428
	TCG ACT GTC CAA ACA TGG TTT AAG CCG CTT GCT TAC GCT TTT ATT CTC	1476
	ACA AGG GAA TCT GGA TAC CCT CAG GTT TTC TAC GGG GAT ATG TAC GGG	1524
50	ACG AAA GGA GAC TCC CAG CGC GAA ATT CCT GCC TTG AAA CAC AAA ATT	1572
	GAA CCG ATC TTA AAA GCG AGA AAA CAG TAT GCG TAC GGA GCA CAG CAT	1620
55	GAT TAT TTC GAC CAC CAT GAC ATT GTC GGC TGG ACA AGG GAA GGC GAC	1668

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	AGC TCG GTT GCA AAT TCA GGT TTG GCG GCA TTA ATA ACA GAC GGA CCC	1716
	GGT GGG GCA AAG CGA ATG TAT GTC GGC CGG CAA AAC GCC GGT GAG ACA	1764
5	TGG CAT GAC ATT ACC GGA AAC CGT TCG GAG CCG GTT GTC ATC AAT TCG	1812
	GAA GGC TGG GGA GAG TTT CAC GTA AAC GGC GGG TCG GTT TCA ATT TAT	1860
10	GTT CAA AGA TAG AAGAGCAGAG AGGACGGATT TCCTGAAGGA AATCCGTTTT	1912
	TTTATTTT	1920
(2) INFORMATION FOR SEQ ID NO: 12:		
15	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 2084 base pairs	
	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
20	(ii) MOLECULE TYPE: DNA (genomic)	
	(ix) FEATURE:	
	(A) NAME/KEY: CDS	
	(B) LOCATION: 343..1794	
25	(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:	
	GCCCCGCACA TACGAAAAGA CTGGCTGAAA ACATTGAGCC TTTGATGACT GATGATTTGG	60
	CTGAAGAAGT GGATCGATTG TTTGAGAAAA GAAGAAGACC ATAAAAATAC CTTGTCTGTC	120
30	ATCAGACAGG GTATTTTTTA TGCTGTCCAG ACTGTCCGCT GTGTAAAAAT AAGGAATAAA	180
	GGGGGGTTGT TATTATTTTA CTGATATGTA AAATATAATT TGTATAAGAA AATGAGAGGG	240
35	AGAGGAAACA TGATTCAAAA ACGAAAGCGG ACAGTTTCGT TCAGACTTGT GCTTATGTGC	300
	ACGCTGTTAT TTGTCAGTTT GCCGATTACA AAAACATCAG CC GTA AAT GGC ACG	354
	CTG ATG CAG TAT TTT GAA TGG TAT ACG CCG AAC GAC GGC CAG CAT TGG	402
40	AAA CGA TTG CAG AAT GAT GCG GAA CAT TTA TCG GAT ATC GGA ATC ACT	450
	GCC GTC TGG ATT CCT CCC GCA TAC AAA GGA TTG AGC CAA TCC GAT AAC	498
45	GGA TAC GGA CCT TAT GAT TTG TAT GAT TTA GGA GAA TTC CAG CAA AAA	546
	GGG ACG GTC AGA ACG AAA TAC GGC ACA AAA TCA GAG CTT CAA GAT GCG	594
	ATC GGC TCA CTG CAT TCC CGG AAC GTC CAA GTA TAC GGA GAT GTG GTT	642
50	TTG AAT CAT AAG GCT GGT GCT GAT GCA ACA GAA GAT GTA ACT GCC GTC	690
	GAA GTC AAT CCG GCC AAT AGA AAT CAG GAA ACT TCG GAG GAA TAT CAA	738
55	ATC AAA GCG TGG ACG GAT TTT CGT TTT CCG GGC CGT GGA AAC ACG TAC	786

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	AGT GAT TTT AAA TGG CAT TGG TAT CAT TTC GAC GGA GCG GAC TGG GAT	834
	GAA TCC CGG AAG ATC AGC CGC ATC TTT AAG TTT CGT GGG GAA GGA AAA	882
5	GCG TGG GAT TGG GAA GTA TCA AGT GAA AAC GGC AAC TAT GAC TAT TTA	930
	ATG TAT GCT GAT GTT GAC TAC GAC CAC CCT GAT GTC GTG GCA GAG ACA	978
10	AAA AAA TGG GGT ATC TGG TAT GCG AAT GAA CTG TCA TTA GAC GGC TTC	1026
	CGT ATT GAT GCC GCC AAA CAT ATT AAA TTT TCA TTT CTG CGT GAT TGG	1074
	GTT CAG GCG GTC AGA CAG GCG ACG GGA AAA GAA ATG TTT ACG GTT GCG	1122
15	GAG TAT TGG CAG AAT AAT GCC GGG AAA CTC GAA AAC TAC TTG AAT AAA	1170
	ACA AGC TTT AAT CAA TCC GTG TTT GAT GTT CCG CTT CAT TTC AAT TTA	1218
20	CAG GCG GCT TCC TCA CAA GGA GGC GGA TAT GAT ATG AGG CGT TTG CTG	1266
	GAC GGT ACC GTT GTG TCC AGG CAT CCG GAA AAG GCG GTT ACA TTT GTT	1314
	GAA AAT CAT GAC ACA CAG CCG GGA CAG TCA TTG GAA TCG ACA GTC CAA	1362
25	ACT TGG TTT AAA CCG CTT GCA TAC GCC TTT ATT TTG ACA AGA GAA TCC	1410
	GGT TAT CCT CAG GTG TTC TAT GGG GAT ATG TAC GGG ACA AAA GGG ACA	1458
30	TCG CCA AAG GAA ATT CCC TCA CTG AAA GAT AAT ATA GAG CCG ATT TTA	1506
	AAA GCG CGT AAG GAG TAC GCA TAC GGG CCC CAG CAC GAT TAT ATT GAC	1554
	CAC CCG GAT GTG ATC GGA TGG ACG AGG GAA GGT GAC AGC TCC GCC GCC	1602
35	AAA TCA GGT TTG GCC GCT TTA ATC ACG GAC GGA CCC GGC GGA TCA AAG	1650
	CGG ATG TAT GCC GGC CTG AAA AAT GCC GGC GAG ACA TGG TAT GAC ATA	1698
40	ACG GGC AAC CGT TCA GAT ACT GTA AAA ATC GGA TCT GAC GGC TGG GGA	1746
	GAG TTT CAT GTA AAC GAT GGG TCC GTC TCC ATT TAT GTT CAG AAA TAA	1794
	GGTAATAAAA AAACACCTCC AAGCTGAGTG CGGGTATCAG CTTGGAGGTG CGTTTATTTT	1854
45	TTCAGCCGTA TGACAAGGTC GGCATCAGGT GTGACAAATA CGGTATGCTG GCTGTCATAG	1914
	GTGACAAATC CGGGTTTTGC GCCGTTTGGC TTTTTCACAT GTCTGATTTT TGTATAATCA	1974
50	ACAGGCACGG AGCCGGAATC TTTCGCCTTG GAAAAATAAG CGGCGATCGT AGCTGCTTCC	2034
	AATATGGATT GTTCATCGGG ATCGCTGCTT TTAATCACAA CGTGGGATCC	2084

55 (2) INFORMATION FOR SEQ ID NO: 13:
(i) SEQUENCE CHARACTERISTICS:

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(A) LENGTH: 1455 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

	CATCATAATG GAACAAATGG TACTATGATG CAATATTTTCG AATGGTATTT GCCAAATGAC	60
10	GGGAATCATT GGAACAGGTT GAGGGATGAC GCAGCTAACT TAAAGAGTAA AGGGATAACA	120
	GCTGTATGGA TCCCACCTGC ATGGAAGGGG ACTTCCCAGA ATGATGTAGG TTATGGAGCC	180
15	TATGATTTAT ATGATCTTGG AGAGTTTAAC CAGAAGGGGA CGGTTCGTAC AAAATATGGA	240
	ACACGCAACC AGCTACAGGC TGCGGTGACC TCTTTAAAAA ATAACGGCAT TCAGGTATAT	300
	GGTGATGTCG TCATGAATCA TAAAGGTGGA GCAGATGGTA CGGAAATTGT AAATGCGGTA	360
20	GAAGTGAATC GGAGCAACCG AAACCAGGAA ACCTCAGGAG AGTATGCAAT AGAAGCGTGG	420
	ACAAAGTTTG ATTTTCCTGG AAGAGGAAAT AACCATTCCA GCTTTAAGTG GCGCTGGTAT	480
25	CATTTTGATG GGACAGATTG GGATCAGTCA CGCCAGCTTC AAAACAAAAT ATATAAATTC	540
	AGGGGAACAG GCAAGGCCTG GGAAGTGGAA GTCGATACAG AGAATGGCAA CTATGACTAT	600
	CTTATGTATG CAGACGTGGA TATGGATCAC CCAGAAGTAA TACATGAACT TAGAAACTGG	660
30	GGAGTGTGGT ATACGAATAC ACTGAACCTT GATGGATTTA GAATAGATGC AGTGAAACAT	720
	ATAAAATATA GCTTTACGAG AGATTGGCTT ACACATGTGC GTAACACCAC AGGTAAACCA	780
35	ATGTTTGCAG TGGCTGAGTT TTGGAAAAAT GACCTTGGTG CAATTGAAAA CTATTTGAAT	840
	AAAACAAGTT GGAATCACTC GGTGTTTGAT GTTCCTCTCC ACTATAATTT GTACAATGCA	900
	TCTAATAGCG GTGGTTATTA TGATATGAGA AATATTTTAA ATGGTTCTGT GGTGCAAAAA	960
40	CATCCAACAC ATGCCGTTAC TTTTGTTGAT AACCATGATT CTCAGCCCGG GGAAGCATTG	1020
	GAATCCTTTG TTCAACAATG GTTTAAACCA CTTGCATATG CATTGGTTCT GACAAGGGAA	1080
45	CAAGGTTATC CTTCCGTATT TTATGGGGAT TACTACGGTA TCCCAACCCA TGGTGTCCG	1140
	GCTATGAAAT CTAAAATAGA CCCTCTTCTG CAGGCACGTC AAACCTTTGC CTATGGTACG	1200
	CAGCATGATT ACTTTGATCA TCATGATATT ATCGGTTGGA CAAGAGAGGG AAATAGCTCC	1260
50	CATCCAAATT CAGGCCTTGC CACCATTATG TCAGATGGTC CAGGTGGTAA CAAATGGATG	1320
	TATGTGGGGA AAAATAAAGC GGGACAAGTT TGGAGAGATA TTACCGGAAA TAGGACAGGC	1380
55	ACCGTCACAA TTAATGCAGA CGGATGGGGT AATTTCTCTG TTAATGGAGG GTCCGTTTCG	1440

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GTTTGGGTGA AGCAA

1455

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 1455 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

	CATCATAATG GGACAAATGG GACGATGATG CAATACTTTG AATGGCACTT GCCTAATGAT	60
	GGGAATCACT GGAATAGATT AAGAGATGAT GCTAGTAATC TAAGAAATAG AGGTATAACC	120
15	GCTATTTGGA TTCCGCCTGC CTGGAAAGGG ACTTCGCAA ATGATGTGGG GTATGGAGCC	180
	TATGATCTTT ATGATTTAGG GGAATTTAAT CAAAAGGGGA CGGTTTCGTAC TAAGTATGGG	240
20	ACACGTAGTC AATTGGAGTC TGCCATCCAT GCTTTAAAGA ATAATGGCGT TCAAGTTTAT	300
	GGGGATGTAG TGATGAACCA TAAAGGAGGA GCTGATGCTA CAGAAAACGT TCTTGCTGTC	360
	GAGGTGAATC CAAATAACCG GAATCAAGAA ATATCTGGGG ACTACACAAT TGAGGCTTGG	420
25	ACTAAGTTTG ATTTTCCAGG GAGGGGTAAT ACATACTCAG ACTTTAAATG GCGTTGGTAT	480
	CATTTCGATG GTGTAGATTG GGATCAATCA CGACAATTCC AAAATCGTAT CTACAAATTC	540
30	CGAGGTGATG GTAAGGCATG GGATTGGGAA GTAGATTCGG AAAATGGAAA TTATGATTAT	600
	TTAATGTATG CAGATGTAGA TATGGATCAT CCGGAGGTAG TAAATGAGCT TAGAAGATGG	660
	GGAGAATGGT ATACAAATAC ATTAAATCTT GATGGATTTA GGATCGATGC GGTGAAGCAT	720
35	ATTAAATATA GCTTTACACG TGATTGGTTG ACCCATGTAA GAAACGCAAC GGGAAAAGAA	780
	ATGTTTGCTG TTGCTGAATT TTGGAAAAT GATTTAGGTG CCTTGAGAA CTATTTAAAT	840
40	AAAACAACT GGAATCATTC TGTCTTTGAT GTCCCCCTTC ATTATAATCT TTATAACGCG	900
	TCAAATAGTG GAGGCAACTA TGACATGGCA AAACCTCTTA ATGGAACGGT TGTTCAAAG	960
	CATCCAATGC ATGCCGTAAC TTTTGTGGAT AATCACGATT CTCAACCTGG GGAATCATTA	1020
45	GAATCATTTG TACAAGAATG GTTTAAGCCA CTTGCTTATG CGCTTATTTT AACAGAGAA	1080
	CAAGGCTATC CCTCTGTCTT CTATGGTGAC TACTATGGAA TTCCAACACA TAGTGTCCTA	1140
50	GCAATGAAAG CCAAGATTGA TCCAATCTTA GAGGCGCGTC AAAATTTTGC ATATGGAACA	1200
	CAACATGATT ATTTTGACCA TCATAATATA ATCGGATGGA CACGTGAAGG AAATACCACG	1260
55	CATCCCAATT CAGGACTTGC GACTATCATG TCGGATGGGC CAGGGGGAGA GAAATGGATG	1320

25

TACGTAGGGC AAAATAAAGC AGGTCAAGTT TGGCATGACA TAACTGGAAA TAAACCAGGA 1380
 ACAGTTACGA TCAATGCAGA TGGATGGGCT AATTTTTCAG TAAATGGAGG ATCTGTTTCC 1440
 5 ATTTGGGTGA AACGA 1455

- (2) INFORMATION FOR SEQ ID NO: 15:
 (i) SEQUENCE CHARACTERISTICS:
 10 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
 15 (A) DESCRIPTION: /desc = "Primer BSG1"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCATGATGCA GTATTTTGAA TGG
 13

20

- (2) INFORMATION FOR SEQ ID NO: 16:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 25 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "Primer BSG3"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

30

GTCACCATAA AAGACGCACG GG
 12

- (2) INFORMATION FOR SEQ ID NO: 17:
 35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 70 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 40 (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "Primer BSGM1"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GTCATAGTTT CCGAATTCCG TGTCTACTTC CCAATCCCAA TCCCAAGCTT
 45 TGCCGCGGAA TTTGTAAATG
 70

- (2) INFORMATION FOR SEQ ID NO: 18:

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26

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 41 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "Primer BSGM2"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

10 CTACTTCCCA ATCCCAAGCT TTGCCGCGGA ATTTGTAAAT G

41

- (2) INFORMATION FOR SEQ ID NO: 19:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 26 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "Primer BSGM3"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GGATGATCCA TGTCAAAGTCG GCATAC

26

25

- (2) INFORMATION FOR SEQ ID NO: 20:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "Primer BSGM4"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CTCGGTCACC ACGTGGGGAT GATCC

25

- (2) INFORMATION FOR SEQ ID NO: 21:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "Primer BSGM5"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

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27

CCAGTTTTTC AGCTGGGTCA CGAC

24

CLAIMS

1. A variant of a parent Termamyl-like α -amylase with α -amylase activity comprising mutations in two, three, four, five or six
 5 of the following regions/positions or in corresponding positions in other parent Termamyl-like α -amylases:

(relative to SEQ ID NO: 1):

1: R181*, G182*, T183*, G184*

2: N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

10 3: V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;

4: E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

5: E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

6: K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

(relative to SEQ ID NO: 2):

15 1: R181*,G182*,D183*,G184*

2: N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

3: V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;

4: E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

5: E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

20 6: K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

(Relative to SEQ ID NO: 3):

1: R179*,G180,I181*,G182*

2: N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

3: L204A,R,D,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;

25 4: E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

5: E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

6: S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V

Relative to SEQ ID NO: 4):

1: N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

30 2: I201A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V;

3: D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

4: E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

5: Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

(relative to SEQ ID NO: 5):

35 1: R176*,G177*,E178,G179*

2: N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

3: V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;

4: D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

5: E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

6: Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

(relative to SEQ ID NO: 6):

5 1: R181*,G182*,H183*,G184*

2: N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

3: I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V;

4: E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

5: E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

10 6: K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

2. The variant according to claim 1, comprising the following mutations: N190F/Q264S in SEQ ID NO: 4 or in corresponding positions in another parent α -amylase.

15

3. The variant according to claim 1, comprising the following mutations: I181*/G182*/N193F in SEQ ID NO: 3 or in corresponding positions in another parent Termamyl like α -amylase.

20

4. The variant according to claim 3, further comprising a substitution in position E214Q in SEQ ID NO: 3 or in a corresponding position in another parent Termamyl like α -amylase.

25

5. The variant according to any of claims 1 to 4, wherein the parent α -amylase is a hybrid α -amylase of SEQ ID NO: 4 and SEQ ID NO: 5.

30

6. The variant according to claim 5, wherein the parent hybrid α -amylase is a hybrid α -amylase comprising the 445 C-terminal amino acid residues of the *B. licheniformis* α -amylase shown in SEQ ID NO: 4 and the 37 N-terminal amino acid residues of the α -amylase derived from *B. amyloliquefaciens* shown in SEQ ID NO: 5.

35

7. The variant according to claim 6, wherein the parent hybrid

Termamyl-like α -amylase further has the following mutations:
H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO:
4).

5 8. The variant according to claim 1, exhibiting increased
stability at acidic pH and/or low Ca^{2+} concentration:

9. A DNA construct comprising a DNA sequence encoding an α -
amylase variant according to any one of claims 1 to 8.

10

10. A recombinant expression vector which carries a DNA con-
struct according to claim 9.

11. A cell which is transformed with a DNA construct according
15 to claim 9 or a vector according to claim 10.

12. A cell according to claim 11, which is a microorganism.

13. A cell according to claim 12, which is a bacterium or a
20 fungus.

14. The cell according to claim 13, which is a grampositive
bacterium such as *Bacillus subtilis*, *Bacillus licheniformis*,
Bacillus lentus, *Bacillus brevis*, *Bacillus stearothermophilus*,
25 *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus*
coagulans, *Bacillus circulans*, *Bacillus lautus* or *Bacillus thu-*
ringiensis.

15. A detergent additive comprising an α -amylase variant accor-
30 ding to any one of claims 1 to 8, optionally in the form of a
non-dusting granulate, stabilised liquid or protected enzyme.

16. A detergent additive according to claim 15 which contains
0.02-200 mg of enzyme protein/g of the additive.

35

17. A detergent additive according to claims 15 or 16, which
additionally comprises another enzyme such as a protease, a

lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

18. A detergent composition comprising an α -amylase variant according to any of claims 1 to 8.

19. The detergent composition according to claim 18 which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

10

20. A manual or automatic dishwashing detergent composition comprising an α -amylase variant according to any one of claims 1 to 8.

15 21. A dishwashing detergent composition according to claim 20 which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

20 22. A manual or automatic laundry washing composition comprising an α -amylase variant according to any one of claims 1 to 8.

23. A laundry washing composition according to claim 22, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, an amylolytic enzyme and/or a cellulase.

25

24. A composition comprising:

(i) a mixture of the α -amylase from *B. licheniformis* having the sequence shown in SEQ ID NO: 4 with one or more variants according to any of claims 1 to 8 derived from (as the parent Termamyl-like α -amylase) the *B. stearothermophilus* α -amylase having the sequence shown in SEQ ID NO: 3; or

(ii) a mixture of the α -amylase from *B. stearothermophilus* having the sequence shown in SEQ ID NO: 3 with one or more variants according to any of claims 1 to 8 derived from one or more other parent Termamyl-like α -amylases; or

35

(iii) a mixture of one or more variants according any of claim 1 to 8 derived from (as the parent Termamyl-like α -amylase) the *B. stearothermophilus* α -amylase having the sequence shown in SEQ ID NO: 3 with one or more variants according to the invention
5 derived from one or more other parent Termamyl-like α -amylases.

25. A composition comprising:
a mixture of one or more variants according any of claims 1 to 8 derived from (as the parent Termamyl-like α -amylase) the *B. stearothermophilus* α -amylase having the sequence shown in SEQ ID
10 NO: 3 and a Termamyl-like alpha-amylase derived from the *B. licheniformis* α -amylase having the sequence shown in SEQ ID NO: 4.

15 26. The composition comprising:
a mixture of one or more variants according any of claims 1 to 8 derived from (as the parent Termamyl-like α -amylase) the *B. stearothermophilus* α -amylase having the sequence shown in SEQ ID NO: 3 and a hybrid alpha-amylase comprising a part of the *B. amyloliquefaciens* α -amylase shown in SEQ ID NO: 5 and a part of
20 the *B. licheniformis* α -amylase shown in SEQ ID NO: 4.

27. The composition according to claim 26, wherein the hybrid α -amylase is a hybrid alpha-amylase comprising the 445 C-terminal
25 amino acid residues of the *B. licheniformis* α -amylase shown in SEQ ID NO: 4 and the 37 N-terminal amino acid residues of the α -amylase derived from *B. amyloliquefaciens* shown in SEQ ID NO: 5.

28. The composition according to claim 27, wherein the hybrid α -
30 amylase further has the following mutations:
H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4).

29. The composition according to claims 26, comprising a mixture

of TVB146 and LE174.

30. Use of an α -amylase variant according to any of claims 1 to 8 or a composition according to any of claims 24 to 29 for washing and/or dishwashing.

31. Use of an α -amylase variant according to any of claims 1 to 8 or a composition according to any of claims 24 to 29 for textile desizing.

10

32. Use of an α -amylase variant according to any of claims 1 to 8 or a composition according to any of claims 24 to 29 for starch liquefaction.

15 33. A method for generating a variant of a parent Termamyl-like α -amylase, which variant exhibits increased stability at low pH and at low calcium concentration relative to the parent, the method comprising:

(a) subjecting a DNA sequence encoding the parent Termamyl-like α -amylase to random mutagenesis,

(b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

(c) screening for host cells expressing a mutated α -amylase which has increased stability at low pH and low calcium concentration relative to the parent α -amylase.

1	HHNGTNGTMM	QYFEWHLPPND	GNHWNRLRDD	ASNLNRNGIT	AIWIPPAWKG	50
1	..NGTNGTMM	QYFEWYLPND	GNHWNRLRSD	ASNLKDKGIS	AVWIPPAWKG	
2	HHNGTNGTMM	QYFEWYLPND	GNHWNRLRDD	AANLKSNGIT	AVWIPPAWKG	
3	VNGTLM	QYFEWYTPND	GQHWKRLQND	AEHLSDIGIT	AVWIPPAYKG	
4	..ANLNGTLM	QYFEWYMPND	GQHWRRRLQND	SAYLAEHGIT	AVWIPPAYKG	
5	.AAPFNGTMM	QYFEWYLPDD	GTLWTKVANE	ANNLSSLGIT	ALWLPPAYKG	
6						
51	TSQNDVGYGA	YDLYDLGFEF	QKGTVRTKYG	TRSQLESAIH	ALKNNGVQVY	100
1	ASQNDVGYGA	YDLYDLGFEF	QKGTIRTKYG	TRNQLQAAVN	ALKSNGIQVY	
2	TSQNDVGYGA	YDLYDLGFEF	QKGTVRTKYG	TRNQLQAQVT	SLKNNGIQVY	
3	LSQSDNGYGP	YDLYDLGFEF	QKGTVRTKYG	TKSELQDAIG	SLHSRNVQVY	
4	TSQADVGYGA	YDLYDLGFEF	QKGTVRTKYG	TKGELQSAIK	SLHSRDINVY	
5	TSRSDVGYGV	YDLYDLGFEF	QKGTVRTKYG	TKAQYLQAIQ	AAHAAGMQVY	
6						
101	GDVVMNHKGG	ADATENVLAV	EVNPNNRNQE	ISGDYTIQAW	TKFDFPGRGN	150
1	GDVVMNHKGG	ADATEMVRV	EVNPNNRNQE	VSGEYTIQAW	TKFDFPGRGN	
2	GDVVMNHKGG	ADGTEIVNAV	EVNRSNRNQE	TSGEYAIQAW	TKFDFPGRGN	
3	GDVVLNKHAG	ADATEDVTAV	EVNPNNRNQE	TSEYQIQA	TDFRFPGRGN	
4	GDVVINHKGG	ADATEDVTAV	EVDPADRNRV	ISGEHLIKAW	THFHPGRGS	
5	ADVVFDPKHGG	ADGTEWVDV	EVNPSDRNQE	ISGTQIQAW	TKFDFPGRGN	
6						
151	TYSDFKWRWY	HFDGVDWDQS	RQFQNRKYF	RGDGKAWDWE	VDSNGNYDY	200
1	THSNFKWRWY	HFDGVDWDQS	RKLNNRKYF	RGDGKAWDWE	VDTEGNYDY	
2	NHSSFKWRWY	HFDGTDWDQS	RQLQNKIYF	RGDGKAWDWE	VDTEGNYDY	
3	TYSDFKWHWY	HFDGADWDES	RKI.SRIKF	RGEKAWDWE	VSSNGNYDY	
4	TYSDFKWHWY	HFDGTDWDES	RKL.NRIKF	.QKAWDWE	VSNENGYDY	
5	TYSSFKWRWY	HFDGVDWDES	RKL.SRIKF	RGIGKAWDWE	VDTEGNYDY	
6						

Fig. 1

5	201	LMYADVDMDH	PEVVNELRRW	GEWYNTLNL	DGERIDAVKH	IKYSFTRDWL	250
1	1	LMYADIDMDH	PEVVNELRNW	GVWYNTLGL	DGERIDAVKH	IKYSFTRDWS	
	2	LMYADVDMDH	PEVIHELNRW	GVWYNTLNL	DGERIDAVKH	IKYSFTRDWL	
	3	LMYADVDMDH	PDVVAETKKW	GIWYANELSL	DGERIDAAKH	IKFSFLRDWV	
	4	LMYADVDYDH	PDVVAETKKW	GIWYANELSL	DGERIDAAKH	IKFSFLRDWV	
10	5	LMYADIDYDH	PDVAAEIKRW	GTWYANELQL	DGERLDAVKH	IKFSFLRDWV	
	6	LMYADLDMDH	PEVVTELKNW	GKWYVNTTNI	DGERLDAVKH	IKFSFFPDWL	
	251	THVRNATGKE	MFAVAEEFWKN	DLGALENYLN	KTNWNHSHVFD	VPLHYNLYNA	300
15	1	IHVRSATGKN	MFAVAEEFWKN	DLGAIENYLN	KTNWNHSHVFD	VPLHYNLYNA	
	2	THVRNTTGKP	MFAVAEEFWKN	DLGAIENYLN	KTSWNHSAFD	VPLHYNLYNA	
	3	QAVRQATGKE	MFTVAEYWQN	NAGKLENYLN	KTSFNQSVFD	VPLHFNQAA	
	4	NHVRREKTGKE	MFTVAEYWQN	DLGALENYLN	KTNFNHSHVFD	VPLHYQFHAA	
	5	SYVRSQTGKP	LFTVGEYWSY	DINKLHNYIT	KTDGTMSLFD	APLHNKFYTA	
20	6						
	301	SNSGGNYDMA	KLNGTVVQK	HPMHAFTFVD	NHDSQPGESL	ESFVQEWFKP	350
	1	SKSGGNYDMR	QIFNGTVVQR	HPMHAFTFVD	NHDSQPEEAL	ESFVEEWFKP	
	2	SNSGGYDDMR	NILNGSVVQK	HPTHAVTFVD	NHDSQPGESL	ESFVQQWFKP	
	3	SSQGGGYDDMR	RLLDGTVVSR	HPEKAVTFVE	NHDTQPGQSL	ESTVQTWFKP	
25	4	STQGGGYDDMR	KLNGTVVSK	HPLKSVTFVD	NHDTQPGQSL	ESTVQTWFKP	
	5	SKSGGAFDMR	TLMTNTLMKD	QPTLAVTFVD	NHDTQPGQAL	QSWVDPWFKP	
	6						
	351	LAYALILTRE	QGYPSVFYGD	YYGIPTHS..	.VPAMKAKID	PILEARQNFA	400
30	1	LAYALTLTRE	QGYPSVFYGD	YYGIPTHG..	.VPAMKSKID	PILEARQKYA	
	2	LAYALVLTRE	QGYPSVFYGD	YYGIPTHG..	.VPAMKSKID	PLLQARQTFE	
	3	LAYAFILTRE	SGYPQVFYGD	MYGKTGTSRK	EIPSLKDNIE	PILKARKEYA	
	4	LAYAFILTRE	SGYPQVFYGD	MYGKTGDSQR	EIPALKHKIE	PILKARKQYA	
35	5	LAYAFILTRQ	EGYPCVFYGD	YYGIPQYN..	.IPSLKSKID	PLLIARRDYA	
	6						

Fig. 1

Figure 1 (continued)

401	YGTQHDYFDH	HNIIGWTREG	NTTHPNSGLA	TIMSDGPGGE	KWMYVVGQNK	450
1	YGRQN.....					
2	YGTQHDYFDH	HDIIGWTREG	NSSHPNSGLA	TIMSDGPGGN	KWMYVVGKNKA	
3	YGPQHDYIDH	PDVIGWTREG	DSSAAKSGLA	ALITDGP GGS	KRMYAGL KNA	
4	YGAQHDYFDH	HDIVGWTREG	DSSVANSGLA	ALITDGP GGA	KRMYVGRQNA	
5	YGTQHDYLDH	SDIIGWTREG	GTEKPGSGLA	ALITDGP GGS	KWMYVVGKQHA	
6						
10	451					500
1	GQVWHDITGN	KPGTVTINAD	GWANFSVNGG	SVSIWVKR..		
2						
3	GQVWRDITGN	RTGTVTINAD	GWGNFSVNGG	SVSVWVKQ..		
4	GETWYDITGN	RSDTVKIGSD	GWGEFHVNDG	SVSIYVQ...		
5	GETWHDITGN	RSEPVVINSE	GWGEFHVNGG	SVSIYVQR..		
6	GKVIFYDLTGN	RSDTVTINSD	GWGEFKVNGG	SVSVWVPRKT	TVSTIARPI T	
15	501					
1		519				
2						
3						
4						
5						
6	TRPWTGEFVR	WTEPRLVAW				
20						
25						

Fig. 1

Figure 1 (continued)