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(54) Title: ALPHA-AMYLASE VARIANT WITH ALTERED PROPERTIES

(57) Abstract: The present invention relates to variants (mutants) of parent Termamyl-like alpha-amylases, which variant has alpha-amylase activity and exhibits altered properties relative to the parent alpha-amylase.



WO 02/092797 A2

Alpha-amylase variant with altered properties

FIELD OF THE INVENTION

The present invention relates to a variant of parent Termamyl-like alpha-amylase, which variant has alpha-amylase activity and exhibits an alteration in at least one of the following properties relative to said parent alpha-amylase:

Substrate specificity, substrate binding, substrate cleavage pattern, thermal stability, pH activity profile, pH stability profile, stability towards oxidation, Ca^{2+} dependency, reduced and increased pl and improved wash performance, specific activity, stability under, e.g., high temperature and/or low pH conditions, in particular at low calcium concentrations. The variant of the invention are suitable for starch conversion, ethanol production, laundry wash, dish wash, hard surface cleaning, textile desizing, and/or sweetener production.

BACKGROUND OF THE INVENTION

Alpha-Amylases (alpha-1,4-glucan-4-glucanohydrolases, E.C. 3.2.1.1) constitute a group of enzymes, which catalyze hydrolysis of starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides.

BRIEF DISCLOSURE OF THE INVENTION

The object of the present invention is to provide a Termamyl-like alpha-amylase, which variant in comparison to the corresponding parent alpha-amylase, i.e., un-mutated alpha-amylase, has alpha-amylase activity and exhibits an alteration in at least one of the above mentioned properties relative to said parent alpha-amylase.

Nomenclature

In the present description and claims, the conventional one-letter and three-letter codes for amino acid residues are used. For ease of reference, alpha-amylase variants of the invention are described by use of the following nomenclature:

Original amino acid(s): position(s): substituted amino acid(s)

According to this nomenclature, for instance the substitution of alanine for asparagine in position 30 is shown as:

Ala30Asn or A30N

a deletion of alanine in the same position is shown as:

Ala30* or A30*

and insertion of an additional amino acid residue, such as lysine, is shown as:

Ala30AlaLys or A30AK

A deletion of a consecutive stretch of amino acid residues, such as amino acid residues 30-33, is indicated as (30-33)* or Δ (A30-N33).

Where a specific alpha-amylase contains a "deletion" in comparison with other alpha-amylases and an insertion is made in such a position this is indicated as:

5 *36Asp or *36D

for insertion of an aspartic acid in position 36.

Multiple mutations are separated by plus signs, i.e.:

Ala30Asp + Glu34Ser or A30N+E34S

10 representing mutations in positions 30 and 34 substituting alanine and glutamic acid for asparagine and serine, respectively.

When one or more alternative amino acid residues may be inserted in a given position it is indicated as

A30N,E or

A30N or A30E

15 Furthermore, when a position suitable for modification is identified herein without any specific modification being suggested, it is to be understood that any amino acid residue may be substituted for the amino acid residue present in the position. Thus, for instance, when a modification of an alanine in position 30 is mentioned, but not specified, it is to be understood that the alanine may be deleted or substituted for any other amino acid, i.e., any one of:

20 R,N,D,A,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

Further, "A30X" means any one of the following substitutions:

A30R, A30N, A30D, A30C, A30Q, A30E, A30G, A30H, A30I, A30L, A30K, A30M, A30F, A30P, A30S, A30T, A30W, A30Y, or A30 V; or in short: A30R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

25 If the parent enzyme - used for the numbering - already has the amino acid residue in question suggested for substitution in that position the following nomenclature is used:

"X30N" or "X30N,V" in the case where for instance one or N or V is present in the wildtype.

Thus, it means that other corresponding parent enzymes are substituted to an "Asn" or "Val" in position 30.

30

Characteristics of amino acid residues

Charged amino acids:

Asp, Glu, Arg, Lys, His

35 Negatively charged amino acids (with the most negative residue first):

Asp, Glu

Positively charged amino acids (with the most positive residue first):

Arg, Lys, His

5 Neutral amino acids:

Gly, Ala, Val, Leu, Ile, Phe, Tyr, Trp, Met, Cys, Asn, Gln, Ser, Thr, Pro

Hydrophobic amino acid residues (with the most hydrophobic residue listed last):

Gly, Ala, Val, Pro, Met, Leu, Ile, Tyr, Phe, Trp,

10

Hydrophilic amino acids (with the most hydrophilic residue listed last):

Thr, Ser, Cys, Gln, Asn

BRIEF DESCRIPTION OF THE DRAWINGS

15

Figure 1 is an alignment of the amino acid sequences of five parent Termamyl-like alpha-amylases. The numbers on the extreme left designate the respective amino acid sequences as follows:

1: SEQ ID NO: 4 (SP722)

2: SEQ ID NO: 2 (SP690)

20

3: SEQ ID NO: 10 (BAN)

4: SEQ ID NO: 8 (BLA)

5: SEQ ID NO: 6 (BSG).

DETAILED DISCLOSURE OF THE INVENTION

25

The object of the present invention is to provide a Termamyl-like amylase, which variant has alpha-amylase activity and exhibits an alteration in at least one of the following properties relative to the parent alpha-amylase: substrate specificity, substrate binding, substrate cleavage pattern, thermal stability, pH activity profile, pH stability profile, stability towards oxidation, Ca²⁺ dependency, specific activity, stability under, e.g., high temperature and/or low pH conditions, in particular at low calcium concentrations.

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Termamyl-like alpha-amylases

A number of alpha-amylases produced by *Bacillus* spp. are highly homologous (identical) on the amino acid level.

35

The identity of a number of known *Bacillus* alpha-amylases can be found in the below Table 1:

Table 1

	Percent identity							
	707	AP137	BAN	BSG	SP690	SP722	AA560	Termamyl
	8							
707	100.0	86.4	66.9	66.5	87.6	86.2	95.5	68.1
AP1378	86.4	100.0	67.1	68.1	95.1	86.6	86.0	69.4
BAN	66.9	67.1	100.0	65.6	67.1	68.8	66.9	80.7
BSG	66.5	68.1	65.6	100.0	67.9	67.1	66.3	65.4
SP690	87.6	95.1	67.1	67.9	100.0	87.2	87.0	69.2
SP722	86.2	86.6	68.8	67.1	87.2	100.0	86.8	70.8
AA560	95.5	86.0	66.9	66.3	87.0	86.8	100.0	68.3
Termamyl	68.1	69.4	80.7	65.4	69.2	70.8	68.3	100.0

For instance, the *B. licheniformis* alpha-amylase (BLA) comprising the amino acid sequence shown in SEQ ID NO: 8 (commercially available as Termamyl™) has been found to be about 81% homologous with the *B. amyloliquefaciens* alpha-amylase comprising the amino acid sequence shown in SEQ ID NO: 10 and about 65% homologous with the *B. stearothermophilus* alpha-amylase (BSG) comprising the amino acid sequence shown in SEQ ID NO: 6. Further homologous alpha-amylases include SP690 and SP722 disclosed in WO 95/26397 and further depicted in SEQ ID NO: 2 and SEQ ID NO: 4, respectively, herein. Other amylases are the AA560 alpha-amylase derived from *Bacillus* sp. and shown in SEQ ID NO: 12, and the #707 alpha-amylase derived from *Bacillus* sp., shown in SEQ ID NO: 13 and described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31.

The KSM AP1378 alpha-amylase is disclosed in WO 97/00324 (from KAO Corporation). Still further homologous alpha-amylases include the alpha-amylase produced by the *B. licheniformis* strain described in EP 0252666 (ATCC 27811), and the alpha-amylases identified in WO 91/00353 and WO 94/18314. Other commercial Termamyl-like alpha-amylases are comprised in the products sold under the following tradenames: Optitherm™ and Takatherm™ (Solvay); Maxamyl™ (available from Gist-brocades/Genencor), Spezym AA™ and Spezyme Delta AA™ (available from Genencor), and Keistase™ (available from Daiwa), Dex lo, GC 521 (available from Genencor) and Ultraphlow (from Enzyme Biosystems).

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

Accordingly, in the present context, the term "Termamyl-like" alpha-amylase" is intended to indicate an alpha-amylase, in particular *Bacillus* alpha-amylase, especially *Bacillus licheniformis* alpha-amylase, which, at the amino acid level, exhibits a substantial identity to Termamyl™, i.e., the *B. licheniformis* alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8,

herein.

In other words, all the following alpha-amylases, which has the amino acid sequences shown in SEQ ID NOS: 2, 4, 6, 8, 10, 12 and 13 herein are considered to be "Termamyl-like alpha-amylase". Other Termamyl-like alpha-amylases are alpha-amylases i) which displays at least 60%, such as at least 70%, e.g., at least 75%, or at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology (identity) with at least one of said amino acid sequences shown in SEQ ID NOS: 2, 4, 6, 8, 10, 12, and 13, and/or is encoded by a DNA sequence which hybridizes to the DNA sequences encoding the above-specified alpha-amylases which are apparent from SEQ ID NOS: 1, 3, 5, 7, 9, and of the present specification (which encoding sequences encode the amino acid sequences shown in SEQ ID NOS: 2, 4, 6, 8, 10 and 12 herein, respectively).

Homology (Identity)

The homology may be determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (described above). Thus, Gap GCGv8 may be used with the default scoring matrix for identity and the following default parameters: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, respectively for nucleic acidic sequence comparison, and GAP creation penalty of 3.0 and GAP extension penalty of 0.1, respectively, for protein sequence comparison. GAP uses the method of Needleman and Wunsch, (1970), J.Mol. Biol. 48, p.443-453, to make alignments and to calculate the identity.

A structural alignment between Termamyl (SEQ ID NO: 8) and, e.g., another alpha-amylase may be used to identify equivalent/corresponding positions in other Termamyl-like alpha-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)).

Hybridisation

The oligonucleotide probe used in the characterisation of the Termamyl-like alpha-amylase above may suitably be prepared on the basis of the full or partial nucleotide or amino acid sequence of the alpha-amylase in question.

Suitable conditions for testing hybridisation involve pre-soaking in 5xSSC and prehybri-

dizing for 1 hour at 40°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 hybridisation in the same solution supplemented with 100 mM ATP for 18 hours at 40°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 75°C (very high stringency). More details about the hybridisation 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 alpha-amylase produced or producible by a strain of the organism in question, but also an alpha-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 alpha-amylase, which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the alpha-amylase in question. The term is also intended to indicate that the parent alpha-amylase may be a variant of a naturally occurring alpha-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 alpha-amylase.

Parent Termamyl-like Alpha-amylases

According to the invention any Termamyl-like alpha-amylase, as defined above, may be used as the parent (i.e., backbone) alpha-amylase. In a preferred embodiment of the invention the parent alpha-amylase is derived from *B. licheniformis*, e.g., one of those referred to above, such as the *B. licheniformis* alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8.

Parent hybrid Termamyl-like Alpha-amylases

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

The parent hybrid alpha-amylase may be one, which on the basis of amino acid homology (identity) and/or DNA hybridization (as defined above) can be determined to belong to the Termamyl-like alpha-amylase family. In this case, the hybrid alpha-amylase is typically composed of at least one part of a Termamyl-like alpha-amylase and part(s) of one or more other alpha-amylases selected from Termamyl-like alpha-amylases or non-Termamyl-like alpha-amylases of microbial (bacterial or fungal) and/or mammalian origin.

Thus, the parent hybrid alpha-amylase may comprise a combination of partial amino acid

sequences deriving from at least two Termamyl-like alpha-amylases, or from at least one Termamyl-like and at least one non-Termamyl-like bacterial alpha-amylase, or from at least one Termamyl-like and at least one fungal alpha-amylase. The Termamyl-like alpha-amylase from which a partial amino acid sequence derives, may be any of the specific Termamyl-like alpha-amylase referred to herein.

For instance, the parent alpha-amylase may comprise a C-terminal part of an alpha-amylase derived from a strain of *B. licheniformis*, and a N-terminal part of an alpha-amylase derived from a strain of *B. amyloliquefaciens* or from a strain of *B. stearothermophilus* (BSG). For instance, the parent alpha-amylase may comprise at least 430 amino acid residues of the C-terminal part of the *B. licheniformis* alpha-amylase, and may, e.g., comprise a) an amino acid segment corresponding to the 37 N-terminal amino acid residues of the *B. amyloliquefaciens* alpha-amylase having the amino acid sequence shown in SEQ ID NO: 10 and an amino acid segment corresponding to the 445 C-terminal amino acid residues of the *B. licheniformis* alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8, or a hybrid Termamyl-like alpha-amylase being identical to the Termamyl sequence, i.e., the *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 8, 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., the *Bacillus amyloliquefaciens* alpha-amylase shown in SEQ ID NO: 10; or b) an amino acid segment corresponding to the 68 N-terminal amino acid residues of the *B. stearothermophilus* alpha-amylase having the amino acid sequence shown in SEQ ID NO: 6 and an amino acid segment corresponding to the 415 C-terminal amino acid residues of the *B. licheniformis* alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8.

Another suitable parent hybrid alpha-amylase is the one previously described in WO 96/23874 (from Novo Nordisk) constituting the N-terminus of BAN, *Bacillus amyloliquefaciens* alpha-amylase (amino acids 1-300 of the mature protein) and the C-terminus from Termamyl (amino acids 301-483 of the mature protein).

In a preferred embodiment of the invention the parent Termamyl-like alpha-amylase is a hybrid alpha-amylase of SEQ ID NO: 8 and SEQ ID NO: 10. Specifically, the parent hybrid Termamyl-like alpha-amylase may be a hybrid alpha-amylase comprising the 445 C-terminal amino acid residues of the *B. licheniformis* alpha-amylase shown in SEQ ID NO: 8 and the 37 N-terminal amino acid residues of the alpha-amylase derived from *B. amyloliquefaciens* shown in SEQ ID NO: 10, which may suitably further have the following mutations: H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 8). The latter mentioned hybrid is used in the examples below and is referred to as LE174.

Other specifically contemplated parent alpha-amylase include LE174 with fewer mutations, i.e., the right above mentioned hybrid having the following mutations:

A181T+N190F+A209V+Q264S; N190F+A209V+Q264S; A209V+Q264S; Q264S;
 H156Y+N190F+A209V+Q264S; H156Y+A209V+Q264S; H156Y+Q264S;
 H156Y+A181T+A209V+Q264S; H156Y+A181T+Q264S; H156Y+Q264S;
 H156Y+A181T+N190F+Q264S; H156Y+A181T+N190F; H156Y+A181T+N190F+A209V. These
 5 hybrids are also considered to be part of the invention.

In a preferred embodiment the parent Termamyl-like alpha amylase is LE174, SP722, or
 AA560 including any of LE174+G48A+T49I+G107A+I201F; LE174+M197L;
 LE174+G48A+T49I+G107A+M197L+I201F, or SP722+D183*+G184*;
 SP722+D183*+G184*+N195F; SP722+D183*+G184*+M202L;
 10 SP722+D183*+G184*+N195F+M202L; BSG+I181*+G182*; BSG+I181*+G182*+N193F;
 BSG+I181*+G182*+M200L; BSG+I181*+G182*+N193F+M200L;
 AA560+D183*+G184*; AA560+D183*+G184*+N195F; AA560+D183*+G184*+M202L;
 AA560+D183*+G184*+N195F+M202L.

"BSG+I181*+G182*+N193F" means the *B. stearothermophilus* alpha-amylase has been
 15 mutated as follows: deletions in positions I181 and G182 and a substitution from Asn (N) to
 Phe (F) in position 193.

Other parent alpha-amylases contemplated include LE429, which is LE174 with an
 additional substitution in I201F. According to the invention LE335 is the alpha-amylase, which in
 comparison to LE429 has additional substitutions in T49I+G107A; LE399 is LE335+G48A, i.e.,
 20 LE174, with G48A+T49I+G107A+I201F.

Altered Properties

The following section describes the relationship between mutations, which are present in a
 variant of the invention, and desirable alterations in properties (relative to those of a parent
 25 Termamyl-like alpha-amylase), which may result therefrom.

As mentioned above the invention relates to a Termamyl-like alpha-amylase with altered
 properties.

Parent Termamyl-like alpha-amylaseS specifically contemplated in connection with going
 through the specifically contemplated altered properties are the above mentioned parent
 30 Termamyl-like alpha-amylase and parent hydrid Termamyl-like alpha-amylases.

The *Bacillus licheniformis* alpha-amylase (SEQ ID NO: 8) is used as the starting point, but
 corresponding positions in, e.g., the SP722, BSG, BAN, AA560, SP690, KSM AP1378, #707
 and other Termmayl-like alpha-amylases should be understood as disclosed and specifically
 contemplated too.

35 In an aspect the invention relates to variant with altered properties as mentioned above.

In the first aspect a variant of a parent Termamyl-like alpha-amylase, comprising an

alteration at one or more positions (using SEQ ID NO: 8 for the amino acid numbering) selected from the group of:

5, 6, 36, 37, 38, 39, 42, 45, 47, 63, 66, 69, 70, 71, 72, 74, 75, 76, 79, 82, 83, 86, 87, 89, 93, 112, 113, 117, 120, 137, 213, 216, 220, 223, 225, 226, 227, 229, 243, 245, 279, 282, 311, 321, 324, 352, 353, 354, 357, 361, 362, 364, 368, 390, 395, 397, 399, 400, 401, 425, 451, 452, 453, 466, 468, 470, 471, 478,

wherein

(a) the alteration(s) are independently

(i) an insertion of an amino acid downstream of the amino acid which occupies the position,

(ii) a deletion of the amino acid which occupies the position, or

(iii) a substitution of the amino acid which occupies the position with a different amino acid, (b) the variant has alpha-amylase activity and (c) each position corresponds to a position of the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8.

In the *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 8 the positions to be mutated are one or more of:

G5, T6, G36, I37, T38, A39, I42, A45, K47, D63, E66, Q69, K70, G71, T72, R74, T75, K76, T79, E82, L83, A86, I87, S89, R93, T112, E113, A117, V120, A137, K213, G216, A220, L223, L225, D226, G227, R229, D243, V245, F279, S282, T311, V321, V324, L352, T353, R354, G357, V361, F362, G364, G368, A390, A395, G397, Q399, H400, D401, A425, D451, I452, T453, G466, G468, F470, H471, S478.

In the first aspect the invention also relates to a variant of a parent Termamyl-like alpha-amylase, comprising a substitution at one or more positions (using SEQ ID NO: 8 for the amino acid numbering) selected from the group of:

7, 8, 9, 11, 12, 19, 21, 22, 25, 32, 40, 41, 46, 48, 55, 57, 58, 60, 77, 95, 97, 98, 99, 100, 101, 102, 103, 105, 107, 115, 118, 135, 139, 141, 143, 151, 159, 160, 161, 162, 163, 166, 175, 177, 183, 186, 187, 192, 199, 200, 202, 203, 208, 212, 215, 219, 228, 230, 233, 236, 238, 240, 241, 244, 248, 256, 258, 259, 260, 262, 270, 273, 274, 277, 281, 283, 284, 285, 286, 287, 288, 289, 292, 295, 296, 304, 307, 312, 313, 320, 322, 323, 325, 326, 478, 327, 329, 331, 339, 343, 344, 346, 347, 349, 350, 359, 360, 369, 377, 380, 387, 409, 410, 411, 412, 423, 424, 426, 427, 428, 429, 430, 438, 440, 441, 449, 462, 472, 477, 479, 480, 481

wherein

(a) the variant has alpha-amylase activity and (b) each position corresponds to a position of the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8.

In the *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 8 the positions to be mutated are one or more of:

L7, M8, Q9, F11, E12, G19, H21, W22, L25, L32, V40, W41, Y46, G48, G55, G57, A58, D60, Y77, I95, V97, Y98, G99, D100, V101, V102, I103, H105, G107, V115, V118, I135, T139, F141,
 5 F143, S151, H159, F160, D161, G162, T163, D166, Y175, F177, D183, V186, S187, N192, A199, D200, D202, Y203, V208, I212, W215, Y219, F228, L230, V233, I236, F238, F240, L241, W244, V248, M256, T258, V259, A260, Y262, L270, Y273, L274, T277, H281, V283, F284, D285, V286, P287, L288, H289, F292, A295, S296, M304, L307, V312, V313, S320, T322, F323, D325, N326, H327, T329, P331, V339, F343, K344, L346, A347, A349, F350, P359,
 10 Q360, T369, I377, L380, I387, V409, G410, W411, T412, G423, S478, L424, A426, L427, I428, T429, D430, M438, V440, G441, W449, I462, V472, V477, I479, Y480, V481

Specific substitutions contemplated are:

X7A,R,N,D,C,Q,E,G,H,K,M,P,S,Y,V;
 X8C,M;
 15 X9A,R,N,D,C,Q,G,H,M,P,S,T,W,Y,V;
 X11A,N,D,C,Q,G,H,I,L,M,P,S,T,W,Y,V;
 X12A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X19A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X21A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 20 X22A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X25A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X32A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X40A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X41A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 25 X46A,R,D,C,G,K,M,P,W,Y;
 X48R,N,D,C,Q,E,G,H,K,M,F,P,W,Y;
 X55A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X57R,N,D,C,Q,E,G,H,K,M,P,W;
 X58A,R,N,D,C,Q,E,G,H,K,M,S,T,W,Y;
 30 X60A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X77A,R,D,C,G,K,M,P,W,Y;
 X95A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X97A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X98A,R,D,C,G,K,M,P,W,Y;
 35 X99R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X100A,R,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;

- X101A,N,C,Q,G,I,L,M,P,S,T,W,Y,V;
X102N,D,C,Q,E,H,I,L,M,F,P,W,Y,V;
X103A,N,D,C,Q,E,G,M,P,S,W,Y;
X105A,N,C,Q,G,H,I,L,M,P,S,T,Y,V;
5 X107R,N,D,Q,E,H,K,M,F,P,W,Y;
X115R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
X118R,N,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
X135A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X139A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
10 X141A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X143A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,Y,V;
X151A,R,N,D,C,Q,E,G,H,K,M,P,S,T,Y,V;
X159A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,V;
X160A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
15 X161A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X162A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X163A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X166A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X175A,R,D,C,G,K,M,P,W,Y;
20 X177A,N,D,C,Q,E,H,I,L,K,M,P,S,T,W,Y,V;
X183A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X186A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X187A,R,C,Q,E,G,H,I,L,K,M,F,P,W,Y,V;
X192A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
25 X199R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X200A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X202A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X203A,R,D,C,G,K,M,P;
X208A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V;
30 X212A,N,C,Q,G,H,M,P,S,T,V;
X215A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X219A,R,D,C,G,K,M,P,W,Y;
X228A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V;
X230A,R,N,D,C,Q,E,G,L,M,P,S,T,W,Y,V;
35 X233R,N,C,Q,E,G,H,I,K,M,P,S,T,W,Y;
X236A,C,Q,G,H,I,M,P,S,T,V;

- X238A,R,N,D,C,Q,E,G,H,I,K,M,P,S,T,W,Y,V;
X240A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X241A,N,C,Q,G,H,P,S,T,V;
X244A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
5 X248A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X256C,M;
X258A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X259A,R,N,D,C,Q,E,G,H,K,M,P,S,T,W,Y,V;
X260R,N,D,C,Q,E,H,I,L,K,M,F,P,T,W,Y,V;
10 X262A,R,D,C,G,K,M,P,W,Y;
X270A,N,C,Q,G,I,L,M,F,P,S,T,W,Y,V;
X273A,R,D,C,G,K,M,P,Y;
X274A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X277A,R,N,D,C,Q,E,G,H,K,M,P,S,W,Y,V;
15 X281A,R,N,D,C,Q,E,G,K,M,P,S,T,W,Y,V;
X283A,R,N,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
X284A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,Y,V;
X285A,R,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
X286A,R,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V; preferably
20 X286N,C,Q,I,L,M,P,T,V,Y,F;
X287R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X288A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X289A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X292A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
25 X295A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X296A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X304C,M;
X307A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X312A,N,C,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
30 X313A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X320R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X322R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V;
X323A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
X325A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
35 X326A,R,N,C,Q,E,G,M,P,S,T,W;
X327A,R,C,G,H,I,L,K,M,P,S,T,W,Y,V;

- X329A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
X331N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X339A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X343A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,Y,V;
5 X344A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X346A,R,N,D,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X347A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X349R,N,D,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X350A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
10 X359R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X360N,D,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X369A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X377A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X380A,R,N,D,C,Q,E,G,H,K,P,S,W,Y,V;
15 X387A,R,N,D,C,Q,E,G,H,L,K,M,P,S,T,W,Y,V;
X409N,C,Q,E,G,H,M,P,S,T,W,Y,V;
X410A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X411A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X412R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
20 X423A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X424A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X426A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X427A,R,N,D,C,Q,E,G,H,K,M,P,S,T,Y,V;
X428A,N,D,Q,E,G,H,I,M,F,P,S,W,Y,V;
25 X429A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X430A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X438C,M;
X440R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X441A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
30 X449A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X462A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X472A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X477A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X479A,R,N,D,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
35 X480A,R,D,C,G,K,M,P,W,Y;
X481A,R,N,D,C,Q,E,G,H,K,M,P,S,T,Y,V

where each position corresponds to a position of the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8 (*Bacillus licheniformis* alpha-amylase).

Also according to the first aspect, the invention relates to a variant of a parent Termamyl-like alpha-amylase, comprising a substitution at one or more positions (using SEQ ID NO: 8 for the amino acid numbering) selected from the group of:

1, 2, 3, 4, 13, 14, 16, 17, 18, 20, 23, 24, 26, 34, 35, 49, 50, 51, 52, 53, 61, 62, 67, 68, 73, 84, 85, 88, 91, 92, 96, 106, 108, 114, 116, 119, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 136, 138, 145, 147, 148, 149, 150, 152, 153, 154, 155, 156, 157, 158, 164, 165, 167, 168, 169, 170, 171, 172, 173, 176, 179, 180, 181, 182, 184, 185, 188, 189, 190, 191, 193, 196, 198, 204, 205, 206, 209, 210, 211, 214, 217, 218, 221, 222, 234, 235, 237, 239, 242, 246, 247, 249, 250, 251, 252, 253, 254, 255, 257, 261, 263, 265, 266, 267, 268, 269, 271, 272, 275, 276, 278, 280, 290, 291, 293, 294, 297, 298, 299, 300, 301, 302, 303, 305, 306, 308, 309, 310, 314, 315, 316, 317, 318, 319, 328, 332, 333, 334, 335, 336, 337, 338, 340, 341, 342, 345, 355, 358, 363, 370, 371, 373, 374, 375, 376, 378, 379, 381, 389, 393, 394, 396, 398, 402, 403, 404, 405, 406, 407, 408, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 431, 432, 433, 434, 435, 436, 437, 439, 442, 443, 444, 445, 446, 447, 448, 450, 454, 455, 456, 457, 458, 459, 460, 461, 463, 464, 465, 467, 469, 473, 474, 475, 476, 482, 483

wherein

(a) the variant has alpha-amylase activity and (b) each position corresponds to a position of the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8.

In the *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 8 the positions to be mutated are one or more of:

A1, N2, L3, N4, W13, Y14, P16, N17, D18, Q20, R23, R24, Q26, E34, H35, T49, S50, Q51, A52, D53, L61, Y62, F67, H68, V73, Q84, S85, K88, H91, S92, N96, K106, G108, D114, T116, E119, D121, P122, A123, D124, R125, N126, R127, V128, I129, S130, G131, E132, H133, L134, K136, W138, G145, G147, S148, T149, Y150, D152, F153, K154, W155, H156, W157, Y158, D164, W165, E167, S168, R169, K170, L171, N172, R173, K176, G179, K180, A181, W182, W184, E185, N188, E189, N190, G191, Y193, L196, Y198, D204, H205, P206, A209, A210, E211, R214, T217, W218, N221, E222, K234, H235, K237, S239, R242, N246, H247, R249, E250, K251, T252, G253, K254, E255, F257, E261, W263, N265, D266, L267, G268, A269, E271, N272, N275, K276, N278, N280, Y290, Q291, H293, A294, T297, Q298, G299, G300, G301, Y302, D303, R305, K306, L308, N309, G310, S314, K315, H316, P317, L318, K319, D328, G332, Q333, S334, L335, E336, S337, T338, Q340, T341, W342, P345, E355, Y358, Y363, K370, G371, S373, Q374, R375, E376, P378, A379, K381, K389, Q393, Y394,

Y396, A398, Y402, F403, D404, H405, H406, D407, I408, R413, E414, G415, D416, S417, S418, V419, A420, N421, S422, G431, P432, G433, G434, A435, K436, R437, Y439, R442, Q443, N444, A445, G446, E447, T448, H450, G454, N455, R456, S457, E458, P459, V460, V461, N463, S464, E465, W467, E469, N473, G474, G475, S476, Q482, R483.

5 Specific substitutions contemplated are:

X1A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;

X2R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;

X3A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;

X4A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

10 X13R,N,D,C,Q,E,G,H,K,M,P,S,T,W;

X14A,R,D,C,G,K,M,P,W;

X16R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;

X17A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

X18A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

15 X20A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

X23A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,W,Y,V;

X24A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

X26A,D,C,E,G,H,I,L,M,F,P,S,T,W,V;

X34A,R,N,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;

20 X35A,R,N,D,C,Q,E,G,H,K,M,F,P,S,T,W,Y,V;

X49A,C,G,H,P,T;

X50A,R,N,C,Q,E,G,H,K,M,F,P,S,W;

X51A,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;

X52A,R,D,C,Q,E,G,H,K,P;

25 X53A,D,C,G,H,K,M,P;

X61A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,Y;

X62A,R,D,C,G,K,M,P,Y;

X67A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,Y,V;

X68A,R,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

30 X73A,R,N,D,C,Q,E,G,H,K,M,P,S,T,W,Y,V;

X84A,R,N,D,C,G,H,I,L,K,M,F,P,S,T,W,Y,V;

X85A,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

X88A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

X91A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

35 X92A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;

X96A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

- X106A,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,Y,V;
X108R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X114A,N,C,Q,E,G,H,K,F,P,S,T,W,Y;
X116A,R,D,C,Q,E,G,H,I,L,M,F,P,S,W,Y,V;
5 X119A,R,N,D,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X121A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X122R,N,Q,G,H,I,L,M,F,S,T,W,Y,V;
X123N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X124N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
10 X125R,N,Q,E,G,I,K,M,F,S,T,W,Y;
X126N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X127A,R,N,D,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X128A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
X129A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
15 X130A,R,N,D,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
X131A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X132R,N,D,C,Q,E,G,H,I,L,K,M,F,S,W,Y;
X133R,N,D,C,M,T,W,V;
X134A,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
20 X136A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X138R,N,D,Q,E,G,I,K,M,P,S,T,W,V;
X145A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,Y,V;
X147A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V;
X148A,R,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
25 X149A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V;
X150A,D,C,G,M,P,W,Y;
X152A,R,N,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
X153A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X154A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
30 X155A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X156A,C,Q,E,G,I,L,M,F,P,S,T,W,V;
X157R,I,L,M,F,P,S,T,W,Y,V;
X158R,M,P,W,Y;
X164R,I,L,M,F,P,S,T,W,Y,V;
35 X165A,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
X167A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

- X168A,R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,V;
X169A,R,N,D,C,Q,E,G,H,M,P,S,W,Y,V;
X170A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X171A,R,N,D,C,Q,E,G,H,K,M,P,S,T,W,Y,V;
5 X172A,N,D,C,Q,E,G,I,L,M,F,P,T,W,Y,V;
X173A,N,D,C,Q,E,G,H,M,P,S,W,Y,V;
X176A,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X179D,C,Q,E,H,I,L,K,M,F,P,W,Y,V;
X180A,G,I,L,M,F,P,W,Y,V;
10 X181G;
X182A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X184I,L,M,F,P,W,Y,V;
X185R,I,L,M,F,P,S,T,W,Y,V;
X188A,R,N,Q,G,H,L,M,F,W,V;
15 X189A,R,N,G,H,I,L,M,F,P,S,T,W,Y,V;
X190N;
X191A,R,N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X193A,R,G,M,P,W,Y;
X196A,N,Q,G,H,I,L,M,P,S,T,W,V;
20 X198A,R,G,M,P,W;
X204R,L,M,F,P,T,W,Y,V;
X205A,G,H,I,L,M,F,P,W,Y,V;
X206R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X209R,P,S,W,Y;
25 X210A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,W,Y,V;
X211E;
X214A,D,C,Q,E,G,I,L,M,F,P,S,T,Y,V;
X217A,R,N,D,C,Q,G,H,I,L,M,F,P,S,T,W,Y;
X218A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
30 X221A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X222A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,W,Y,V;
X234A,D,C,G,H,I,K,M,F,P,S,T,W,Y,V;
X235A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X237A,G,H,I,L,M,F,W,Y,V;
35 X239G,H,I,L,M,F,P,S,T,Y,V;
X242G,I,L,M,F,S,T,W,Y,V;

- X246A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X247R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,V;
X249A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X250A,R,N,D,C,E,H,I,L,K,M,P,T,W,Y,V;
5 X251R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X252A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
X253R,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y;
X254A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X255A,R,D,C,G,H,I,L,K,M,F,S,T,W,Y,V;
10 X257A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X261A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X263A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
X265C,Q,E,H,I,L,M,F,P,W;
X266A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
15 X267A,R,N,D,C,Q,E,G,H,K,P,S,T,W,Y,V;
X268A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X269A,N,C,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X271A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X272A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
20 X275A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
X276A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X278A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X280A,R,D,C,E,G,H,I,L,K,M,F,P,W,Y,V;
X290W,Y;
25 X291A,R,N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X293A,R,N,G,I,L,M,P,S,T,W,V;
X294R,N,Q,G,H,I,L,M,F,P,S,T,W,Y;
X297A,G,H,I,L,M,F,P,W,Y,V;
X298G,H,I,L,M,F,P,S,T,W,Y,V;
30 X299A,G,H,M,P,S,T;
X300A,C,G,H,I,L,M,F,P,T,W,Y,V;
X301N,Q,H,I,L,M,F,P,S,T,W,Y,V;
X302R,M,P,W,Y;
X303R,I,L,M,F,P,S,T,W,Y,V;
35 X305G,I,L,M,F,P,S,T,W,Y,V;
X306Q,G,H,I,L,M,F,P,S,T,W,Y,V;

- X308A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X309N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X310A,R,N,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
X314A,D,C,E,G,I,L,M,F,P,W,Y,V;
5 X315A,N,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X316A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,V;
X317R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X318A,R,N,D,C,Q,E,G,H,I,K,P,S,W,Y,V;
X319A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
10 X328A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X332A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X333A,N,D,C,G,I,M,F,P,S,T,Y,V;
X334R,N,C,Q,E,G,H,K,M,F,P,S,W,Y;
X335R,D,C,Q,E,H,I,L,K,M,F,P,W,Y,V;
15 X336A,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X337A,R,N,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X338A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X340I,L,M,F,P,S,T,W,Y,V;
X341A,R,D,C,G,H,I,L,K,M,F,W,Y,V;
20 X342R,I,L,M,F,P,S,T,W,Y,V;
X345R,N,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
X355A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X358A,R,D,C,G,K,M,P,W,Y;
X363A,R,D,C,G,K,M,P,W,Y;
25 X370A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X371A,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X373A,R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;
X374A,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;
X375A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,V;
30 X376A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X378R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;
X379A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
X381A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X389A,D,C,G,H,M,F,P,S,T,W,Y,V;
35 X393A,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X394A,R,D,C,G,K,M,P,W,Y;

- X396A,R,D,C,G,K,M,P,W,Y;
X398A,R,N,D,C,Q,E,G,H,I,L,M,F,W,Y,V;
X402R,C,G,K,M,P,W,Y;
X403A,R,N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
5 X404R,I,L,M,F,P,S,T,W,Y,V;
X405R,G,H,I,L,M,F,P,W,Y,V;
X406A,R,G,H,I,M,F,P,Y,V;
X407A,R,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X408A,R,N,D,C,Q,E,G,H,I,K,M,P,S,T,W,Y,V;
10 X413A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X414A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X415A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X416A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X417A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
15 X418A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X419A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X420A,N,D,C,E,G,H,I,L,K,M,F,S,T,W,Y,V;
X421A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
X422A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
20 X431A,R,N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X432G,H,I,L,M,F,P,S,T,W,Y,V;
X433A,R,N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X434A,R,N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X435Q,G,H,I,L,M,F,P,T,W,Y,V;
25 X436A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X437A,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X439A,R,D,C,G,K,M,P,W,Y;
X442A,R,N,D,C,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X443A,R,N,D,C,E,G,H,I,L,M,F,P,S,T,W,Y,V;
30 X444A,C,G,H,I,L,M,F,P,S,T,W,Y,V;
X445A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X446A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X447A,N,D,C,G,H,I,L,M,F,P,S,T,W,Y,V;
X448A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
35 X450A,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,V;
X454A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

X455A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X456A,D,C,E,G,H,I,L,M,F,P,S,T,W,Y,V;
 X457A,R,N,D,C,Q,E,G,H,I,L,K,M,F,W,Y,V;
 X458A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 5 X459R,N,D,C,Q,E,G,H,I,L,K,M,F,S,W,Y,V;
 X460A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X461A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,W,Y,V;
 X463A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
 X464A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 10 X465A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X467A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X469A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X473N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 X474A,R,G,H,I,L,M,F,P,W,Y,V;
 15 X475A,N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 X476G,H,I,L,M,F,P,S,T,W,Y,V;
 X482A,N,D,C,G,H,I,L,M,F,S,T,W,Y,V;
 X483A,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V

where each position corresponds to a position of the amino acid sequence of the parent
 20 Termamyl-like alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8
 (*Bacillus licheniformis* alpha-amylase).

Specifically contemplated according to the invention is substitution at position
 183 and/or 184 (using SEQ ID NO: 2 (SP690) for the amino acid numbering) with any amino
 acid, i.e. any one of A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y or V.

25 Also in the first aspect the invention relates to a variant of a parent Termamyl-like alpha-
 amylase, comprising an alteration at one or more positions (using SEQ ID NO: 8 for the amino
 acid numbering) selected from the group of:

1, 3, 4, 17, 18, 20, 23, 24, 28, 56, 61, 62, 67, 68, 80, 81, 84, 85, 91, 92, 106, 110, 114, 119, 121,
 122, 123, 124, 125, 126, 127, 129, 131, 134, 136, 172, 185, 196, 206, 217, 218, 231, 232, 235,
 30 246, 247, 249, 251, 257, 278, 310, 316, 317, 328, 332, 355, 358, 363, 367, 370, 373, 375, 376,
 381, 382, 391, 396, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 439, 445, 446, 448, 450,
 454, 455, 458, 459, 460, 461, 463, 464, 465, 467

wherein

(a) the alteration(s) are independently

35 (i) an insertion of an amino acid downstream of the amino acid which occupies the
 position, or

(ii) a deletion of the amino acid which occupies the position,
(b) the variant has alpha-amylase activity and (c) each position corresponds to a position of the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8.

5 In the *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 8 the positions to be mutated are one or more of:

A1, L3, N4, N17, D18, Q20, R23, R24, D28, Y56, L61, Y62, F67, H68, K80, G81, Q84, S85,
H91, S92, K106, D110, D114, E119, D121, P122, A123, D124, R125, N126, R127, I129, G131,
L134, K136, N172, E185, L196, P206, T217, W218, D231, A232, H235, N246, H247, R249,
10 K251, F257, N278, G310, H316, P317, D328, G332, E355, Y358, Y363, Y367, K370, S373,
R375, E376, K381, H382, R391, Y396, R413, E414, G415, D416, S417, S418, V419, A420,
N421, S422, Y439, A445, G446, T448, H450, G454, N455, E458, P459, V460, V461, N463,
S464, E465, W467.

Specific insertions/deletions contemplated are:

15 A1 insertion;
L3 insertion;
N4 insertion;
N17 insertion;
D18 insertion;
20 Q20 insertion;
R23 insertion;
R24 insertion;
D28 insertion;
Y56 insertion;
25 L61 insertion or deletion;
Y62 insertion;
F67 insertion or deletion;
H68 insertion;
K80 insertion or deletion;
30 G81 insertion or deletion;
Q84 insertion;
S85 insertion;
H91 insertion or deletion;
S92 insertion or deletion;
35 K106 insertion or deletion;
D110 insertion or deletion;

D114 deletion;
E119 insertion or deletion;
D121 insertion;
P122 insertion;
5 A123 insertion;
D124 insertion;
R125 insertion;
N126 insertion;
R127 insertion;
10 I129 insertion;
G131 insertion;
L134 insertion;
K136 insertion;
N172 insertion;
15 E185 insertion;
L196 insertion or deletion;
P206 insertion or deletion;
T217 insertion;
W218 insertion;
20 D231 insertion or deletion;
A232 insertion or deletion;
H235 insertion or deletion;
N246 insertion;
H247 insertion;
25 R249 insertion;
K251 insertion;
F257 insertion or deletion;
N278 insertion;
G310 insertion or deletion;
30 H316 insertion;
P317 insertion;
D328 insertion or deletion;
G332 insertion or deletion;
E355 insertion or deletion;
35 Y358 insertion;
Y363 insertion;

- Y367 insertion;
K370 insertion;
S373 insertion;
R375 insertion;
5 E376 insertion;
K381 insertion;
H382 insertion;
R391 insertion or deletion;
Y396 insertion;
10 R413 insertion or deletion;
E414 insertion or deletion;
G415 insertion or deletion;
D416 insertion;
S417 insertion;
15 S418 insertion;
V419 insertion;
A420 insertion;
N421 insertion;
S422 insertion or deletion;
20 Y439 insertion;
A445 insertion or deletion;
G446 insertion or deletion;
T448 insertion or deletion;
H450 insertion;
25 G454 insertion or deletion;
N455 insertion;
E458 insertion;
P459 insertion;
V460 insertion;
30 V461 insertion;
N463 insertion;
S464 insertion;
E465 insertion;
W467 insertion
- 35 where each position corresponds to a position of the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8

(*Bacillus licheniformis* alpha-amylase).

Also in the first aspect the invention relates to a variant of a parent Termamyl-like alpha-amylase, comprising an alteration at one or more positions (using SEQ ID NO: 8 for the amino acid numbering) selected from the group of:

5 7, 8, 10, 11, 12, 15, 19, 21, 22, 25, 40, 41, 43, 44, 46, 55, 59, 77, 78, 90, 95, 97, 98, 99, 100, 101, 102, 105, 109, 115, 118, 135, 139, 141, 195, 208, 215, 219, 236, 238, 240, 244, 248, 256, 258, 259, 312, 313, 320, 322, 323, 325, 326, 327, 330, 331, 348, 349, 350, 359, 360, 365, 366, 369, 377, 384, 388, 423, 424, 438, 441, 449, 462, 479, 480, 481

wherein

10 (a) the alteration(s) are independently

(i) an insertion of an amino acid downstream of the amino acid which occupies the position, or

(ii) a deletion of the amino acid which occupies the position,

(b) the variant has alpha-amylase activity and (c) each position corresponds to a position of the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8.

In the *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 8 the positions to be mutated are one or more of:

20 L7, M8, Y10, F11, E12, M15, G19, H21, W22, L25, V40, W41, P43, P44, Y46, G55, Y59, Y77, G78, L90, I95, V97, Y98, G99, D100, V101, V102, H105, A109, V115, V118, I135, T139, F141, Y195, V208, W215, Y219, I236, F238, F240, W244, V248, M256, T258, V259, V312, V313, S320, T322, F323, D325, N326, H327, Q330, P331, Y348, A349, F350, P359, Q360, D365, M366, T369, I377, I384, L388, G423, L424, M438, G441, W449, I462, I479, Y480, V481.

Specific insertions/deletions contemplated are:

25 L7 insertion or deletion;

M8 insertion;

Y10 insertion;

F11 insertion;

E12 insertion or deletion;

30 M15 insertion;

G19 insertion;

H21 insertion;

W22 insertion;

L25 insertion;

35 V40 insertion or deletion;

W41 insertion;

P43 insertion or deletion;
P44 insertion or deletion;
Y46 insertion;
G55 insertion;
5 Y59 insertion;
Y77 insertion;
G78 insertion or deletion;
L90 insertion or deletion;
I95 insertion;
10 V97 insertion;
Y98 insertion;
G99 insertion;
D100 insertion;
V101 insertion;
15 V102 insertion;
H105 insertion or deletion;
A109 insertion or deletion;
V115 insertion or deletion;
V118 insertion or deletion;
20 I135 insertion;
T139 insertion or deletion;
F141 insertion or deletion;
Y195 insertion;
V208 insertion or deletion;
25 W215 insertion;
Y219 insertion;
I236 insertion or deletion;
F238 insertion or deletion;
F240 insertion or deletion;
30 W244 insertion;
V248 insertion;
M256 insertion;
T258 insertion or deletion;
V259 insertion or deletion;
35 V312 insertion or deletion;
V313 insertion or deletion;

- S320 insertion;
T322 insertion or deletion;
F323 insertion or deletion;
D325 insertion or deletion;
5 N326 insertion;
H327 insertion or deletion;
Q330 insertion or deletion;
P331 insertion or deletion;
Y348 insertion;
10 A349 insertion or deletion;
F350 insertion or deletion;
P359 insertion or deletion;
Q360 insertion;
D365 insertion or deletion;
15 M366 insertion;
T369 insertion;
I377 insertion;
I384 insertion or deletion;
L388 insertion or deletion;
20 G423 insertion or deletion;
L424 insertion or deletion;
M438 insertion;
G441 insertion or deletion;
W449 insertion;
25 I462 insertion;
I479 insertion or deletion;
Y480 insertion;
V481 insertion or deletion;
where each position corresponds to a position of the amino acid sequence of the parent
30 Termamyl-like alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8
(*Bacillus licheniformis* alpha-amylase).

Corresponding positions in other parent alpha-amylases can be found by alignment as described above and shown in the alignment in Fig. 1.

35 Stability

In the context of the present invention, mutations (including amino acid substitutions and

deletion) of importance with respect to achieving altered stability, in particular improved stability (i.e., higher or lower), at especially high temperatures (i.e., 70-120°C) and/or extreme pH (i.e. low or high pH, i.e. pH 4-6 or pH 8-11, respectively), in particular at free (i.e., unbound, therefore in solution) calcium concentrations below 60 ppm, include any of the mutations listed in the
5 "Altered Properties" section. The stability may be determined as described in the "Materials & Methods" section below.

Ca²⁺ stability

Altered Ca²⁺ stability means the stability of the enzyme under Ca²⁺ depletion has been
10 improved, i.e., higher or lower stability. In the context of the present invention, mutations (including amino acid substitutions and deletions) of importance with respect to achieving altered Ca²⁺ stability, in particular improved Ca²⁺ stability, i.e., higher or lower stability, at especially high pH (i.e., pH 8-10.5) include any of the mutations listed in the in "Altered properties" section.

15

Specific activity

In a further aspect of the present invention, important mutations (including amino acid substitutions and deletions) with respect to obtaining variants exhibiting altered specific activity, in particular increased or decreased specific activity, especially at temperatures from 10-60°C,
20 preferably 20-50°C, especially 30-40°C, include any of the mutations listed in the in "Altered properties" section. The specific activity may be determined as described in the "Material & Methods" section below.

Oxidation stability

25 Variants of the invention may have altered oxidation stability, in particular higher oxidation stability, in comparison to the parent alpha-amylase. Increased oxidation stability is advantageous in, e.g., detergent compositions and decreased oxidation stability may be advantageous in composition for starch liquefaction. Oxidation stability may be determined as described in the "Material & Methods" section below.

30

Altered pH profile

Important positions and mutations with respect to obtaining variants with altered pH profile, in particular improved activity at especially high pH (i.e., pH 8-10.5) or low pH (i.e., pH 4-6) include mutations of amino residues located close to the active site residues.

Preferred specific mutations/substitutions are the ones listed above in the section "Altered Properties" for the positions in question. Suitable assays are described in the "Materials & Methods" section below.

5 Wash performance

Important positions and mutations with respect to obtaining variants with improved wash performance at especially high pH (i.e., pH 8.5-11) include the specific mutations/substitutions listed above in the section "Altered Properties" for the positions in question. The wash performance may be tested as described below in the "Materials & Methods" section.

10

Increased pI

Substitutions resulting in higher pI

In an aspect the invention relates to a variant with a higher pI than the parent alpha-amylase. Such variants are suitable when adjusting the pI to the washing conditions of various detergents. This means that if the pI of the parent alpha-amylase is below the pH in the washing solution the target is to increase the pI to the pH of the washing solution. Such variant may be prepared by making the following kind of substitutions:

15

1) Substituting one or more of the below mentioned negatively charged amino acid residue in a parent alpha-amylase with a positively charged amino acid residue.

20

2) Substituting one or more of the below mentioned neutral amino acid residue in a parent alpha-amylase with a positively charged amino acid residue;

3) Substituting one or more of the below mentioned negatively charged amino acid residue in a parent alpha-amylase with a neutral amino acid residue;

25

4) Substituting one or more of the below mentioned positively charged amino acid residue in a parent alpha-amylase with a more positively charged amino acid residue;

Variants of the invention with increased pI in comparison to the parent alpha-amylase may have improved wash performance. Wash performance tests may be carried out as described in the "Materials & Method" section.

Thus, variants of the invention include (using SEQ ID NO: 8 for the numbering):

30

G5R,K,H;

T6R,K,H;

G36R,K,H;

I37R,K,H;

T38R,K,H;

35

A39R,K,H;

I42R,K,H;
A45R,K,H;
D63A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
E66A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
5 Q69R,K,H;
G71R,K,H;
T72R,K,H;
T75R,K,H;
T79R,K,H;
10 E82A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L83R,K,H;
A86R,K,H;
I87R,K,H;
S89R,K,H;
15 T112R,K,H;
E113A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
A117R,K,H;
V120R,K,H;
A137R,K,H;
20 G216R,K,H;
A220R,K,H;
L223R,K,H;
L225R,K,H;
D226A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
25 G227R,K,H;
D243A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
V245R,K,H;
F279R,K,H;
S282R,K,H;
30 T311R,K,H;
V321R,K,H;
V324R,K,H;
L352R,K,H;
T353R,K,H;
35 G357R,K,H;
V361R,K,H;

F362R,K,H;
G364R,K,H;
G368R,K,H;
A390R,K,H;
5 A395R,K,H;
G397R,K,H;
Q399R,K,H;
H400R,K,H;
D401A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
10 A425R,K,H;
D451A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
I452R,K,H;
T453R,K,H;
G466R,K,H;
15 G468R,K,H;
F470R,K,H;
S478R,K,H.
L7R,K,H;
Q9R,H,;
20 F11H;
E12A,R,N,C,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G19R,K,H;
W22R,K,H;
L25R,K,H;
25 L32R,K,H;
V40R,K,H;
W41R,K,H;
Y46R,K;
G48R,K,H;
30 G55R,K,H;
G57R,K,H;
A58R,K,H;
D60A,R,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Y77R,K;
35 I95R,K,H;
V97R,K,H;

Y98R,K,;
G99R,K,H;
D100A,R,C,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
V102H;
5 G107R,K,H;
V115R,K,H;
V118R,K,H;
I135R,K,H;
T139R,K,H;
10 F141R,K,H;
F143R,K,H;
S151R,K,H;
F160R,K,H;
D161R,K,H;
15 G162R,K,H;
T163R,K,H;
D166A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Y175R,K,;
F177K,H;
20 D183A,R,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
V186R,K,H;
S187R,K,H;
N192R,K,H;
A199R,K,H;
25 D200A,R,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D202A,R,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Y203R,K;
V208R,K,H;
I212H;
30 W215R,K,H;
Y219R,K;
F228R,K,H;
L230R;
V233R,K,H;
35 I236H;
F238R,K,H;

- F240R,K,H;
L241H;
W244R,K,H;
V248R,K,H;
5 T258R,K,H;
V259R,K,H;
A260R,K,H;
Y262R,K;
Y273R,K;
10 L274R,K,H;
T277R,K,H;
H281R,K;
V283R,K;
F284RK;
15 D285A,R,C,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
V286R,K,H;
P287R,H;
L288R,K,H;
H289R,K;
20 F292R,K,H;
A295R,K,H;
S296R,K,H;
L307R,K,H;
V312H;
25 V313R,K,H;
S320R,K,H;
T322R,K,H;
F323R,K;
D325A,R,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
30 N326R;
T329R,K,H;
P331H;
V339R,H,K;
F343R,H,K;
35 K344H;
L346R,K,H;

A347R,K,H;
A349R,K,H;
F350R,K,H;
P359R,K,H;
5 Q360H;
T369R,K,H;
I377R,K,H;
L380R,K,H;
I387R,K,H;
10 V409H;
G410R,K,H;
W411R,K,H;
T412R,K,H;
G423R,K,H;
15 L424R,K,H;
A426R,K,H;
L427R,K,H;
I428H;
T429R,K,H;
20 D430A,R,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
V440R,K,H;
G441R,K,H;
W449R,K,H;
I462R,K,H;
25 V472R,K,H;
V477R,K,H;
I479R,K,H;
Y480R,K;
V481R,K,H.
30 A1R,K,H;
N2R,K,H;
L3R,K,H;
N4R,K,H;
W13R,K,H;
35 Y14R,K;
P16R,K,H;

- N17R,K,H;
D18A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Q20R,K,H;
R23H;
5 R24K,H;
Q26H;
E34A,R,N,C,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
T49H;
S50R,K,H;
10 Q51H;
A52R,K,H;
D53A,C,G,H,K,M,P;
L61R,K,H;
Y62R,K;
15 F67R,K,H;
V73R,K,H;
Q84R,K,H;
S85R,K,H;
K88H;
20 S92R,H;
N96R,K,H;
K106H;
G108R,K,H;
D114A,N,C,Q,G,H,K,F,P,S,T,W,Y;
25 T116R,H;
E119A,R,N,D,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D121A,R,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
P122R,H;
A123H;
30 D124N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
R125K;
N126H;
R127K,H;
V128R,K,H;
35 I129R,K,H;
S130R,K,H;

G131R,K,H;
E132R,N,D,C,Q,G,H,I,L,K,M,F,S,W,Y;
L134K,H;
K136A,R,N,D,C,E,G,H,I,L,M,F,P,S,T,W,Y,V;
5 W138R,K;
G145R,K,H;
G147R,K,H;
S148R,K,H;
T149R,K,H;
10 D152A,R,N,C,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
F153R,K,H;
K154H;
W155R,K,H;
W157R;
15 Y158R;
D164R,I,L,M,F,P,S,T,W,Y,V;
W165K,H;
E167A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
S168R,K,H;
20 R169H;
K170H;
L171R,K,H;
R173H;
K176H;
25 G179K,H;
W182R,K,H;
E185R,I,L,M,F,P,S,T,W,Y,V;
N188R,H;
E189A,R,N,G,H,I,L,M,F,P,S,T,W,Y,V;
30 G191R,H;
Y193R;
L196H;
Y198R;
D204R,L,M,F,P,T,W,Y,V;
35 P206R,K,H;
A209R;

- A210R,K,H;
T217R,H;
W218R,K,H;
N221R,K,H;
5 E222R,K,H;
K234H;
K237H;
S239H;
N246R,K,H;
10 R249H;
E250A,R,N,D,C,H,I,L,K,M,P,T,W,Y,V;
K251H;
T252R,K,H;
G253R,K,H;
15 K254H;
E255A,R,D,C,G,H,I,L,K,M,F,S,T,W,Y,V;
F257R,K,H;
E261A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W263R,K;
20 N265H;
D266A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L267R,K,H;
G268R,K,H;
A269H;
25 E271A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
N272R,K,H;
N275R,K,H;
K276H;
N278R,K,H;
30 N280R,K,H;
Q291R,H;
A294R,H;
T297H;
Q298H;
35 G299H;
G300H;

G301H;
Y302R;
D303R,I,L,M,F,P,S,T,W,Y,V;
K306H;
5 L308R,K,H;
N309H;
G310R,K,H;
K315H;
P317R,K,H;
10 L318R,K,H;
K319H;
D328A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G332R,K,H;
S334R,K,H;
15 L335R,K,H;
E336A,N,D,C,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
S337R,H;
T338R,K,H;
T341R,K,H;
20 W342R;
P345R,K,H;
E355A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Y358R,K;
Y363R,K;
25 K370H;
G371K,H;
S373R,K,H;
Q374K,H;
R375H;
30 E376A,R,N,D,C,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
P378R,K,H;
A379R,K,H;
K381H;
K389H;
35 Q393K,H;
Y394R,K;

- Y396R,M;
A398R,H;
Y402R,K;
F403R,H;
5 D404R,I,L,M,F,P,S,T,W,Y,V;
H406R;
D407A,R,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
I408R,K,H;
R413K,H;
10 E414A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G415R,K,H;
D416A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
S417R,K,H;
S418R,K,H;
15 V419R,H;
A420K,H;
N421R,K,H;
S422R,K,H;
G431R,H;
20 P432H;
G433R,H;
G434R;
A435H;
K436H;
25 R437K,H;
Y439R,K;
R442H;
Q443R,H;
N444H;
30 A445R,K,H;
G446R,K,H;
E447A,N,D,C,G,H,I,L,M,F,P,S,T,W,Y,V;
T448R,K,H;
G454R,K,H;
35 N455R,K,H;
R456H;

S457R,K,H;
 E458A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 P459R,K,H;
 V460R,K,H;
 5 V461R,H;
 N463R,K,H;
 S464R,K,H;
 E465A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 W467R,K,H;
 10 E469A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 N473H;
 G474R,H;
 G475H;
 S476H;
 15 Q482H;
 R483H.

Decreased pI

Substitutions resulting in lower pI

20 Variants of the invention with decreased pI in comparison to the parent alpha-amylase may have improved liquefying effect. This means that the pI of the parent amylase should be adjusted to the pH conditions in the liquefying process in question. Normally the pH during liquefaction lies in the range of 4-7, such as between pH 4.5-6.5. An example of a liquefaction process is described below in the section "Liquefaction". Improved liquefying effect may be

25 carried out as described in the "Materials & Method" section.

Alternatively, variants with decreased pI may advantageously be used in detergent. If the pI of the parent alpha-amylase is above the pH in the washing solution the target is to decrease the pI to the pH of the washing solution. Such variant may be prepared by making the following kind of substitutions:

- 30 1) Substituting one or more of the below mentioned positively charged amino acid residue in a parent alpha-amylase with a neutral amino acid residue.
- 2) Substituting one or more of the below mentioned neutral amino acid residue in a parent alpha-amylase with a negatively charged amino acid residue.
- 3) Substituting one or more of the below mentioned positively charged amino acid residue
- 35 with a negatively charged amino acid residue.
- 4) Substituting one or more of the below mentioned negatively charged amino acid residue

with a more negatively charged amino acid residue.

Thus, variants of the invention include (using SEQ ID NO: 8 for the numbering):

- G5D,E;
- T6D,E;
- 5 G36D,E;
- I37D,E;
- T38D,E;
- A39D,E;
- I42D,E;
- 10 A45D,E;
- K47A,R,N,D,C,Q,E,G,H,,M,F,P,S,T,W,Y,V;
- D63E;
- Q69D,E;
- K70A,N,D,C,Q,E,G,I, M,F,P,S,T,W,Y,V;
- 15 G71D,E;
- T72D,E;
- R74A,N,D,C,Q,E,G,I, M,F,P,S,T,W,Y,V;
- T75D,E;
- K76A,N,D,C,Q,E,G,L,M,F,P,S,T,W,Y,V;
- 20 T79D,E;
- L83D,E;
- A86D,E;
- I87D,E;
- S89D,E;
- 25 R93A,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
- T112D,E;
- A117D,E;
- V120D,E;
- A137D,E;
- 30 K213A,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
- G216D,E;
- A220D,E;
- L223D,E;
- L225D,E;
- 35 D226E;
- G227D,E;

R229A,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
D243E;
V245D,E;
F279D,E;
5 S282D,E;
T311D,E;
V321D,E;
V324D,E;
L352D,E;
10 T353D,E;
R354A,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
G357D,E;
V361D,E;
F362D,E;
15 G364D,E;
G368D,E;
A390D,E;
A395D,E;
G397D,E;
20 Q399D,E;
H400A,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
D401E;
A425D,E;
D451E;
25 I452D,E;
T453D,E;
G466D,E;
G468D,E;
F470D,E;
30 H471A,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
S478D,E.
L7D,E;
Q9D;
F11D;
35 G19D,E;
H21R,D,E,K;

W22D,E;
L25D,E;
L32D,E;
V40D,E;
5 W41D,E;
Y46D;
G48D,E;
G55D,E;
G57D,E;
10 A58D,E;
D60E;
Y77D;
I95D,E;
V97D,E;
15 Y98D;
G99D,E;
D100E;
V102D,E;
I103A,D,E;
20 H105A,N,C,Q,G,I,L,M,P,S,T,Y,V;
G107D,E;
V115D,E;
V118E;
I135D,E;
25 T139D,E;
F141D,E;
F143AD,E;
S151D,E;
H159A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,V;
30 F160D,E;
D161E;
G162D,E;
T163D,E;
Y175D;
35 F177D,E;
D183E;

V186D,E;
S187E;
N192D,E;
A199D,E;
5 D200E;
D202E;
Y203D;
V208D,E;
W215D,E;
10 Y219D;
F228D,E;
L230D,E;
V233E;
F238D,E;
15 F240D,E;
W244D,E;
V248D,E;
T258D,E;
V259D,E;
20 A260D,E;
Y262D;
Y273D;
L274D,E;
T277D,E;
25 H281A,R,N,D,C,Q,E,G,K,M,P,S,T,W,Y,V;
V283E;
F284D,E;
D285E;
V286D,E;
30 P287D,E;
L288D,E;
H289A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
F292D,E;
A295D,E;
35 S296D,E;
L307D,E;

V313D,E;
S320D,E;
T322D,E;
F323D,E;
5 D325E;
N326E;
H327A,R,C,G,I,L,K,M,P,S,T,W,Y,V;
T329D,E;
P331D,E;
10 V339E;
F343D,E;
K344A,R,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
L346D,E;
A347D,E;
15 A349D,E;
P359D,E;
Q360D;
T369D,E;
I377D,E;
20 L380D,E;
I387D,E;
V409E;
G410D,E;
W411D,E;
25 T412E;
G423D,E;
L424D,E;
A426D,E;
L427D,E;
30 I428D,E;
T429D,E;
D430E;
V440D,E;
G441D,E;
35 W449D,E;
I462D,E;

- V472D,E;
V477D,E;
I479D,E;
Y480D;
5 V481D,E.
A1D,E;
N2D,E;
L3D,E;
N4D,E;
10 W13D,E;
Y14D;
P16D,E;
N17D,E;
D18E;
15 Q20D,E;
R23D,E;
R24D,E;
Q26D,E;
H35A,R,N,D,C,Q,E,G,K,M,F,P,S,T,W,Y,V;
20 S50E;
Q51D,E;
A52D,E;
L61D,E;
Y62D;
25 F67D,E;
H68A,R,D,C,E,G,I,L,K,M,F,P,S,T,W,Y,V;
V73D,E;
Q84D;
S85E;
30 K88A,R,N,D,C,E,G,I,L,M,F,P,S,T,W,Y,V;
H91A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
S92D,E;
N96D,E;
K106A,N,D,C,Q,E,G,I,L,M,P,S,T,Y,V;
35 G108D,E;
D114E;

- T116D,E;
D121E;
A123D,E;
R125N,Q,E,G,I,M,F,S,T,W,Y;
5 R127D,E;
V128D;
I129D,E;
S130D,E;
G131D;
10 H133R,N,D,C,M,T,W,V;
L134D,E;
K136A,R,N,D,C,E,G,I,L,M,F,P,S,T,W,Y,V;
W138D,E;
G145D,E;
15 G147D,E;
S148D,E;
T149D,E;
Y150D;
D152E;
20 F153D,E;
K154A,R,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
W155D,E;
H156A,C,Q,E,G,I,L,M,F,P,S,T,W,V;
W165D,E;
25 S168D,E;
R169D,E;
K170A,R,N,D,C,E,G,I,L,M,F,P,S,T,W,Y,V;
L171D,E;
N172D,E;
30 R173A,N,D,C,Q,E,G,M,P,S,W,Y,V;
K176A,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
G179D,E;
K180A,G,I,L,M,F,P,W,Y,V;
W182D,E;
35 P206D,E;
A210D,E;

- R214A,D,C,Q,E,G,I,L,M,F,P,S,T,Y,V;
T217D;
W218D,E;
N221D,E;
- 5 K234A,D,C,G,I,M,F,P,S,T,W,Y,V;
H235A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
K237A,G,I,L,M,F,W,Y,V;
R242G,I,L,M,F,S,T,W,Y,V;
N246D,E;
- 10 H247R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,V;
R249A,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
K251R,N,D,C,E,G,I,L,M,F,P,S,T,W,Y,V;
T252D,E;
G253E;
- 15 K254A,R,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
F257D,E;
W263D,E;
N265E;
D266E;
- 20 L267D,E;
G268D,E;
N272D,E;
N275D,E;
K276A,R,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
- 25 N278D,E;
N280D,E;
H293A,R,N,G,I,L,M,P,S,T,W,V;
R305G,I,L,M,F,P,S,T,W,Y,V;
K306Q,G,I,L,M,F,P,S,T,W,Y,V;
- 30 L308D,E;
G310E;
S314D,E;
K315A,N,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
H316A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,V;
- 35 P317D,E;
L318D,E;

K319A,R,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
D328E;
G332E;
Q333D;
5 S334E;
L335D,E;
S337E;
T341D;
P345D,E;
10 Y358D;
Y363D;
K370A,R,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
G371E;
S373D,E;
15 Q374D,E;
R375A,N,D,C,Q,G,I,L,M,F,P,S,T,W,V;
P378D,E;
A379D,E;
K381A,R,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
20 K389A,D,C,G,M,F,P,S,T,W,Y,V;
Q393E;
Y394D;
Y396D;
A398D,E;
25 H405R,G,I,L,M,F,P,W,Y,V;
H406A,R,G,I,M,F,P,Y,V;
I408D,E;
R413A,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
G415D,E;
30 D416E;
S417D,E;
S418D,E;
V419D,E;
A420D,E;
35 N421D,E;
S422D,E;

K436A,R,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
 R437A,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V;
 Y439D;
 R442A,N,D,C,E,G,I,L,M,F,P,S,T,W,Y,V;
 5 Q443D,E;
 A445D,E;
 G446D,E;
 T448D,E,;
 H450A,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,V;
 10 G454D,E;
 R456A,D,C,E,G,I,L,M,F,P,S,T,W,Y,V;
 S457D,E;
 P459D,E;
 V460D,E;
 15 V461D,E;
 N463D,E;
 S464D,E;
 W467D,E;
 Q482D;
 20 R483A,N,D,C,Q,E,G,I,L,M,F,P,S,T,W,Y,V.

Reduced sensitivity to anionic surfactants

Substitutions resulting in a more hydrophilic amino acid residue

In an aspect, the invention relates to providing alpha-amylase variants with reduced
 25 sensitivity (or improved stability against denaturation) to anionic surfactants (in particular
 linear alkyl sulphonates (LAS)). These variants are provided by substituting, deleting or
 inserting an amino acid residue in the parent alpha-amylase as indicated below with a more
 hydrophilic amino acid residue. Such variants may be prepared by:

- 1) Substituting one or more of the below mentioned positively charged amino acid residue in a
 30 parent alpha-amylase with a hydrophilic amino acid residue.
- 2) Substituting one or more of the below mentioned hydrophobic amino acid residue in a
 parent alpha-amylase with a hydrophilic amino acid residue
- 3) Substituting one or more of the below mentioned positively charged amino acid residue in a
 parent alpha-amylase with a netral or negatively charged amino acid residue.

35 The anionic surfactants (in particular linear alkyl sulphonates (LAS)) sensitivity (in
 detergent) may be tested as described in the "Materials & Methods" section.

Variants of the invention with reduced sensitivity to anionic surfactants, in particular linear alkyl sulphonates (LAS), include (using the *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 8 numbering):

- G5N,C,Q,S,T;
5 T6N,C,Q,S;
G36N,C,Q,S,T;
I37N,C,Q,S,T;
T38N,C,Q,S;
A39N,C,Q,S,T;
10 I42N,C,Q,S,T;
A45N,C,Q,S,T;
K47N,C,Q,S,T, A,I,L,M,F,P,W,Y,V, D,E;
Q69N;
K70N,C,Q,S,T, A,I,L,M,F,P,W,Y,V, D,E;
15 G71N,D,C,Q,S,T;
T72N,C,Q,S;
R74N,C,Q,S,T, A,I,L,M,F,P,W,Y,V, D,E;
T75N,C,Q,S;
K76N,C,Q,S,T, A,I,L,M,F,P,W,Y,V, D,E;
20 T79N,C,Q,S;
L83N,C,Q,S,T;
A86N,C,Q,S,T;
I87N,C,Q,S,T;
S89N,C,Q;
25 R93N,C,Q,S,T, A,I,L,M,F,P,W,Y,V, D,E;
T112N,C,Q,S;
A117N,C,Q,S,T;
V120N,C,Q,S,T;
A137N,C,Q,S,T;
30 K213N,C,Q,S,T, A,I,L,M,F,P,W,Y,V, D,E;
G216N,C,Q,S,T;
A220N,C,Q,S,T;
L223N,C,Q,S,T;
L225N,C,Q,S,T;
35 G227N,C,Q,S,T;
R229N,C,Q,S,T, A,I,L,M,F,P,W,Y,V, D,E;

- V245N,C,Q,S,T;
F279N,C,Q,S,T;
S282N,C,Q;
T311N,,C,Q,S;
5 V321N,C,Q,S,T;
V324N,C,Q,S,T;
L352N,C,Q,S,T;
T353N,C,Q,S;
R354N,C,Q,S,T, A,I,L,M,F,P,W,Y,V, D,E;
10 G357N,C,Q,S,T;
V361N,C,Q,S,T;
F362N,C,Q,S,T;
G364N,C,Q,S,T;
G368N,C,Q,S,T;
15 A390N,C,Q,S,T;
A395N,C,Q,S,T;
G397N,C,Q,S,T;
Q399N;
H400N,C,Q,S,T, A,I,L,M,F,P,W,Y,V, D,E;
20 A425N,C,Q,S,T;
I452N,C,Q,S,T;
T453N,C,Q,S;
G466N,C,Q,S,T;
G468N,C,Q,S,T;
25 F470N,C,Q,S,T;
H471N,C,Q,S,T, A,I,L,M,F,P,W,Y,V, D,E;
S478N,C,Q.
L7N,C,Q,S;
M8C;
30 Q9N;
F11N,C,Q,S,T;
G19N,C,Q,S,T;
H21N,C,Q,S,T, A,I,L,M,F,P,W,Y,V, D,E;
W22N,C,Q,S,T;
35 L25N,C,Q,S,T;
L32N,C,Q,S,T;

V40N,C,Q,S,T;
W41N,C,Q,S,T;
Y46C;
G48N,C,Q;
5 G55N,C,Q,S,T;
G57N,C,Q;
A58N,C,Q,S,T;
Y77C;
I95N,C,Q,S,T;
10 V97N,C,Q,S,T;
Y98C;
G99N,C,Q,S,T;
V101N,C,Q,S,T;
V102N,C,Q;
15 I103N,C,Q,S;
H105N,C,Q,S,T, A,I,L,M,P,Y,V,;
G107N,Q;
V115N,C,Q,S,T;
V118N,C,Q,S,T;
20 I135N,C,Q,S,T;
T139N,C,Q,S;
F141N,C,Q,S,T;
F143N,C,Q,S,T;
S151N,C,Q;
25 H159N,C,Q,S,T, A,I,L,M,F,P,W,Y,V,D,E;
F160N,C,Q,S,T;
G162N,C,Q,S,T;
T163N,C,Q,S;
Y175C;
30 F177N,C,Q,S,T;
V186N,C,Q,S,T;
S187C,Q;
A199N,C,Q,S,T;
Y203C;
35 V208N,C,Q,S,T;
I212N,C,Q,S,T;

W215N,C,Q,S,T;
Y219C;
F228N,C,Q,S,T;
L230N,C,Q,S,T;
5 V233N,C,Q,S,T;
I236C,Q,S,T;
F238N,C,Q,S,T;
F240N,C,Q,S,T;
L241N,C,Q,S,T;
10 W244N,C,Q,S,T;
V248N,C,Q,S,T;
M256C;
T258N,C,Q,S;
V259N,C,Q,S,T;
15 A260N,C,Q,T;
Y262C;
L270N,C,Q,S,T;
Y273C;
L274N,C,Q,S,T;
20 T277N,C,Q,S;
H281N,C,Q,S,T, A,M,P,W,Y,V, D,E;
V283N,C,Q,S,T;
F284N,C,Q,S,T;
V286C,Q,S,T;
25 P287N,C,Q,S,T;
L288N,C,Q,S,T;
H289N,C,Q,S,T, A,I,L,M,F,P,W,Y,V,D,E;
F292N,C,Q,S,T;
A295N,C,Q,S,T;
30 S296N,C,Q,T;
M304C;
L307N,C,Q,S,T;
V312N,C,Q,S,T;
V313N,C,Q,S,T;
35 S320N,C,Q;
T322N,C,Q,S;

F323N,C,Q,S,T;
H327C,S,T, A,I,L,M,P,W,Y,V;
T329N,C,Q;
P331N,C,Q,S,T;
5 V339N,C,Q,S,T;
F343N,C,Q,S,T;
K344N,C,Q,S,T;
L346N,Q,S,T;
A347N,C,Q,S,T;
10 A349N,Q,S,T;
F350N,C,Q,S,T;
P359N,C,Q,S,T;
Q360N;
T369N,C,Q,S;
15 I377N,C,Q,S,T;
L380N,C,Q,S;
I387N,C,Q,S,T;
V409N,C,Q,S,T;
G410N,C,Q,S,T;
20 W411N,C,Q,S,T;
T412N,C,Q,S;
G423N,C,Q,S,T;
L424N,C,Q,S,T;
A426N,C,Q,S,T;
25 L427N,C,Q,S,T;
I428N,Q,S;
T429N,C,Q,S;
M438C;
V440N,C,Q,S,T;
30 G441N,C,Q,S,T;
W449N,C,Q,S,T;
I462N,C,Q,S,T;
V472N,C,Q,S,T;
V477N,C,Q,S,T;
35 I479N,Q,S;
Y480C;

V481N,C,Q,S,T.
A1N,C,Q,S,T;
L3N,C,Q,S,T;
N4C,Q,S,T;
5 W13N,C,Q,S,T;
Y14C;
P16N,C,Q,S,T;
Q20N;
R23N,C,Q,S, A,I,L,M,F,P,W,Y,V,D,E;
10 R24N,C,Q,S,T, A,I,L,M,F,P,W,Y,V,D,E;
H35N,C,Q,S,T, A,M,F,P,W,Y,V,D,E;
T49C;
S50N,C,Q;
Q51N;
15 A52C,Q;
L61N,C,Q,S,T;
Y62C;
F67N,C,Q,S,T;
H68C,S,T, A,I,L,M,F,P,W,Y,V,D,E;
20 V73N,C,Q,S,T;
Q84N;
S85N,C,T;
K88N,C,S,T, A,I,L,M,F,P,W,Y,V,D,E;
H91N,C,Q,S,T, A,I,L,M,F,P,W,Y,V,D,E;
25 S92N,C,Q;
K106N,C,Q,S,T, A,I,L,M,F,P,W,Y,V,D,E;
G108N,C,Q,S,T;
T116C,Q,S;
E119N,Q,S,T;
30 P122N,Q,S,T;
A123N,C,Q,S,T;
R125N,Q,S,T, I,M,F,W,Y,E;
R127N,S,T, A,I,L,M,F,P,W,Y,V,D,E;
V128N,C,Q,S;
35 I129N,C,Q,S;
S130N,Q;

- G131N,C,Q,S,T;
H133N,C,T,M,W,V,D;
L134N,C,S,T;
K136N,C,S,T A,I,L,M,F,P,W,Y,V,D,E;
5 W138N,Q,S,T;
G145N,C,Q,S,T;
G147N,C,Q,S,T;
S148C,Q,T;
T149N,C,Q,S;
10 Y150C;
F153N,C,Q,S,T;
K154N,C,Q,S,T A,I,L,M,F,P,W,Y,V,D,E;
W155N,C,Q,S,T;
H156C,Q,S,T A,I,L,M,F,P,W,V,E;
15 W157S,T;
W165N,C,Q,S,T;
S168N,C,Q;
R169AN,C,Q,S A,M,P,W,Y,V,D,E;
K170N,C,S,T A,I,L,M,F,P,W,Y,V,D,E;
20 L171N,C,Q,S,T;
R173N,C,Q,S A,M,P,W,Y,V,D,E;
K176N,C,Q,S,T A,I,L,M,F,P,W,Y,V,D,E;
G179C,Q;
W182N,C,Q,S,T;
25 K180A,I,L,M,F,P,W,Y,V;
W182N,C,Q,S,T, A,I,L,M,F,P,W,Y,V,D,E;
W184I,L,M,F,P,W,Y,V;
G191N,Q,S,T;
L196N,Q,S,T;
30 P206N,C,Q,S,T;
A209S;
A210N,C,Q;
R214C,Q,S,T A,I,L,M,F,P,W,Y,V,D,E;
T217N,C,Q,S;
35 W218N,C,Q,S,T;
K234C,S,T A,I,M,F,P,W,Y,V,D;

- H235N,C,Q,S,T A,I,L,M,F,P,W,Y,V;
R242S,T,I,L,M,F,W,Y,V;
H247N,C,Q,S,T,I,L,M,F,P,W,V;
R249N,C,Q,S,T A,I,L,M,F,P,W,Y,V,D,E;
5 K251N,C,S,T I,L,M,F,P,W,Y,V,D,E;
T252N,C,Q,S;
G253C,Q,S,T;
K254N,C,Q,S,T A,I,L,M,F,P,W,Y,V,D,E;
F257N,C,Q,S,T;
10 W263N,C,Q,S,T;
L267N,C,Q,S,T;
G268N,C,Q,S,T;
A269N,C,Q,S,T;
K276N,C,Q,S,T A,I,L,M,F,P,W,Y,V,D,E;
15 Q291N;
H293N,S,T A,I,L,M,P,W,V;
A294N,Q,S,T;
G299S,T;
G300C,T;
20 G301N,Q,S,T;
R305S,T,I,L,M,F,P,W,Y,V;
K306Q,S,T,I,L,M,F,P,W,Y,V,;
L308N,C,Q,S,T;
G310N,C,Q,S,T;
25 S314C;
K315N,C,Q,S,T A,I,L,M,F,P,W,Y,V,E;
H316N,C,Q,S,T A,I,L,M,F,P,W,V,D,E;
P317N,C,Q,S,T;
L318N,C,Q,S;
30 K319N,C,Q,S,T A,I,L,M,F,P,W,Y,V,D,E;
G332N,C,Q,S,T;
Q333N;
S334N,C,Q;
L335C,Q;
35 S337N,C,Q;
T338N,C,Q,S;

- T341C;
W342S,T;
P345N,C,Q,T;
E355N,C,Q,S,T;
5 Y358C;
Y363C;
K370N,C,Q,S,T A,I,L,M,F,P,W,Y,V,D,E;
G371N,C,Q,S,T;
S373N,C,Q,T;
10 Q374N;
R375N,C,Q,S,T A,I,L,M,F,P,W,V,D;
P378N,C,Q,S,T;
A379N,C,Q,,T;
K381N,C,Q,S,T A,I,L,M,F,P,W,Y,V,D,E;
15 K389C,S,T,A,M,F,P,W,Y,V,D;
Q393N;
Y394C;
Y396C;
A398N,C,Q;
20 Y402C;
F403N,Q,S,T;
H405I,L,M,F,P,W,Y,V;
H406A,I,M,F,P,Y,V;
I408N,C,Q,S,T;
25 R413N,C,Q,S,T A,I,L,M,F,P,W,Y,V,D,E;
G415N,C,Q,S,T;
S417N,Q;
S418N,Q;
V419N,C,Q,S,T;
30 A420N,C,S,T;
S422N,Q;
G431N,Q,S,T;
P432S,T;
G433N,Q,S,T;
35 G434N,Q,S,T;
A435Q,T;

K436N,C,Q,S,T,A,I,L,M,F,P,W,Y,V,D,E;
 R437N,C,Q,S,T,A,I,L,M,F,P,W,Y,V,D,E;
 Y439C;
 R442N,C,S,T, A,I,L,M,F,P,W,Y,V,D,E;
 5 Q443N;
 A445N,C,Q,S,T;
 G446N,C,Q,S,T;
 T448N,C,Q,S;
 H450N,C,Q,S,T, A,I,L,M,F,P,W,V,D,E;
 10 G454N,C,Q,S,T;
 R456C,S,T,A,I,L,M,F,P,W,Y,V,D,E;
 S457N,C,Q;
 P459N,C,Q,S;
 V460N,C,Q,S,T;
 15 V461N,C,Q,S;
 S464N,C,Q,T;
 W467N,C,Q,S,T;
 G475N,Q,S,T;
 Q482N;
 20 R483N,C,Q,S,T,A,I,L,M,F,P,W,Y,V,D,E.

Increased stability at low pH and/or at high temperature

Substitutions resulting in a more hydrophobic amino acid residue

In an aspect the invention relates to Termamyl-like alpha-amylase variant with
 25 increased stability at acidic pH and/or at high temperature in comparison to the parent alpha-
 amylase. Such variants are especially suitable for starch liquefaction.

In the context of the invention the term "acidic pH" means a 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.

30 In the context of the invention "high temperature" is a temperature in the range from 60-
 110°C.

Such variant are variant having a substitution resulting in a more hydrophobic amino
 acid residues. Providing such variant of the invention may be prepared by

- 1) Substituting a charged amino acid residue with a hydrophobic amino acid residue;
- 35 2) Substituting a hydrophilic amino acid residue with a hydrophobic amino acid residue;
- 3) Substituting a hydrophilic amino acid residue with a more hydrophilic amino acid residue;

4) Substituting a hydrophilic amino acid residue with a less hydrophilic amino acid residue.

Thus, variants of the invention include (using SEQ ID NO: 8 for the numbering):

- G5A,V,P,M,L,I,Y,F,W;
T6G,A,V,P,M,L,I,Y,F,W;
5 G36A,V,P,M,L,I,Y,F,W;
I37Y,F,W;
T38G,A,V,P,M,L,I,Y,F,W;
A39V,P,M,L,I,Y,F,W;
I42Y,F,W;
10 A45V,P,M,L,I,Y,F,W;
K47G,A,I,L,M,F,P,W,Y,V;
D63 G,A,V,P,M,L,I,Y,F,W;
E66G,A,V,P,M,L,I,Y,F,W;
Q69T,S,C, G,A,V,P,M,L,I,Y,F,W;
15 K70G,A,V,P,M,L,I,Y,F,W;
G71A,V,P,M,L,I,Y,F,W;
T72G,A,V,P,M,L,I,Y,F,W;
R74G,A,V,P,M,L,I,Y,F,W;
T75G,A,V,P,M,L,I,Y,F,W;
20 K76G,A,V,P,M,L,I,Y,F,W;
T79G,A,V,P,M,L,I,Y,F,W;
E82 G,A,V,P,M,L,I,Y,F,W;
L83I,Y,F,W;
A86V,P,M,L,I,Y,F,W;
25 I87Y,F,W;
S89G,A,V,P,M,L,I,Y,F,W;
R93 G,A,V,P,M,L,I,Y,F,W;
T112 G,A,V,P,M,L,I,Y,F,W;
E113 G,A,V,P,M,L,I,Y,F,W;
30 A117,V,P,M,L,I,Y,F,W;
V120P,M,L,I,Y,F,W;
A137V,P,M,L,I,Y,F,W;
K213G,A,V,P,M,L,I,Y,F,W;
G216A,V,P,M,L,I,Y,F,W;
35 A220V,P,M,L,I,Y,F,W;
L223I,Y,F,W;

L225I,Y,F,W;
D226G,A,V,P,M,L,I,Y,F,W;
G227G,A,V,P,M,L,I,Y,F,W;
R229G,A,V,P,M,L,I,Y,F,W;
5 D243G,A,V,P,M,L,I,Y,F,W;
V245P,M,L,I,Y,F,W;
F279W;
S282T,G,A,V,P,M,L,I,Y,F,W;
T311G,A,V,P,M,L,I,Y,F,W;
10 V321P,M,L,I,Y,F,W;
V324P,M,L,I,Y,F,W;
L352I,Y,F,W;
T353G,A,V,P,M,L,I,Y,F,W;
R354G,A,V,P,M,L,I,Y,F,W;
15 G357A,V,P,M,L,I,Y,F,W;
V361P,M,L,I,Y,F,W;
F362W;
G364A,V,P,M,L,I,Y,F,W;
G368A,V,P,M,L,I,Y,F,W;
20 A390V,P,M,L,I,Y,F,W;
A395V,P,M,L,I,Y,F,W;
G397A,V,P,M,L,I,Y,F,W;
Q399T,S,C,G,A,V,P,M,L,I,Y,F,W;
H400G,A,V,P,M,L,I,Y,F,W;
25 D401G,A,V,P,M,L,I,Y,F,W;
A425V,P,M,L,I,Y,F,W;
D451G,A,V,P,M,L,I,Y,F,W;
I452Y,F,W;
T453G,A,V,P,M,L,I,Y,F,W;
30 G466G,A,V,P,M,L,I,Y,F,W;
G468A,V,P,M,L,I,Y,F,W;
F470W;
H471G,A,V,P,M,L,I,Y,F,W;
S478T,G,A,V,P,M,L,I,Y,F,W
35 Q9A,C,G,M,P,S,T,W,Y,V;
F11W;

E12A,G,I,L,M,F,P,W,Y,V;
G19A,I,L,M,F,P,W,Y,V;
H21A,G,I,L,M,F,P,W,Y,V;
L25I,W,Y;
5 L32I,F,W,Y;
V40I,L,M,F,P,W,Y;
Y46W;
G48M,F,P,W,Y;
G55AI,L,M,F,P,W,Y,V;
10 G57M,P,W;
A58G,M,W,Y;
D60AG,I,L,M,F,P,W,Y,V;
Y77W;
I95F,W,Y;
15 V97I,L,M,F,P,W,Y;
Y98W;
G99I,L,M,F,P,W,Y,V;
D100A,G,I,L,M,F,P,W,Y,V;
V101I,L,M,P,W,Y;
20 V102I,L,M,F,P,W,Y;
I103W;
H105A,G,I,L,M,P,Y,V;
G107M,F,P,W,Y;
V115I,L,M,F,P,W,Y;
25 V118I,L,M,F,P,W,Y;
I135F,W,Y;
T139AG,I,L,M,F,P,W,Y,V;
F141W;
S151A,G,M,P,T,Y,V;
30 H159A,G,I,L,M,F,P,W,V;
F160W;
D161A,G,I,L,M,F,P,W,Y,V;
G162A,I,L,M,F,P,W,Y,V;
T163A,G,I,L,M,F,P,Y,V;
35 D166A,G,I,L,M,F,P,W,Y,V;
Y175W;

F177W;
D183A,G,I,L,M,F,P,W,Y,V;
V186I,L,M,F,P,W,Y;
S187A,G,I,L,M,F,P,W,Y,V;
5 N192A,G,I,L,M,F,P,W,Y,V;
A199G,I,L,M,F,P,W,Y,V;
D200A,G,I,L,M,F,P,W,Y,V;
D202A,G,I,L,M,F,P,W,Y,V;
V208L,M,F,P,W,Y;
10 Y219W;
F228W;
L230W,Y;
V233G,I,M,P,W,Y;
I236M,P;
15 F238W;
F240W;
L241P;
V248A,G,I,L,M,F,P,W,Y;
T258A,G,I,M,F,P,W,Y,V;
20 V259M,P,W,Y;
A260I,L,M,F,P,W,Y,V;
Y262W;
L270I,F,W,Y;
L274I,M,F,P,W,Y;
25 T277A,G,M,P,W,Y,V;
H281A,G,M,P,W,Y,V;
V283I,L,M,F,P,W,Y;
D285A,G,I,M,F,P,W,Y,V;
V286I,M,F,P,W,Y;
30 P287I,L,M,F,W,Y;
L288I,F,W,Y;
H289A,G,I,L,M,F,P,W,Y,V;
F292W;
A295I,L,M,F,P,W,Y,V;
35 S296A,G,I,L,M,F,P,W,T,Y,V;
L307I,F,W,Y;

- V312I,L,M,F,P,W,Y;
V313I,L,M,F,P,W,Y;
S320G,I,L,M,F,P,T,W,Y,V;
T322G,L,M,F,P,W,Y,V;
5 F323W;
D325AG,I,L,M,F,P,W,Y,V;
N326A,C,G,M,P,S,T,W;
H327A,G,I,L,M,P,W,Y,V;
T329A,G,I,L,M,F,P,W,Y,V;
10 P331I,L,M,F,W,Y;
V339I,L,M,F,P,W,Y;
K344AG,I,L,M,F,P,W,Y,V;
L346I,F,W,Y;
A347I,L,M,F,P,W,Y,V;
15 A349I,L,M,F,P,W,Y,V;
P359I,L,M,F,W,Y;
Q360I,L,M,F,P,S,T,W,Y,V;
T369A,G,I,L,M,F,P,W,Y,V;
I377F,W,Y;
20 L380W,Y;
I387W,Y;
V409M,P,W,Y;
G410A,I,L,M,F,P,W,Y,V;
T412G,I,L,M,F,P,W,Y,V;
25 G423A,I,L,M,F,P,W,Y,V;
L424I,,F,W,Y;
A426I,L,M,F,P,W,Y,V;
L427Y;
I428F,W,Y;
30 T429A,G,I,L,M,F,P,W,Y,V;
D430A,G,I,L,M,F,P,W,Y,V;
V440I,L,M,F,P,W,Y;
G441A,I,L,M,F,P,W,Y,V;
I462F,W,Y;
35 V472I,L,M,F,P,W,Y;
V477I,L,M,F,P,W,Y;

- I479F,W,Y;
Y480W;
V481M,P,Y.
A1I,L,M,F,P,W,Y;
5 N2C,Q,I,L,M,F,S,T,W,Y,V;
L3I,M,F,P,W,Y;
N4A,C,Q,I,L,M,F,P,S,T,W,Y,V;
Y14W;
P16I,L,M,F,W,Y;
10 N17A,C,Q,G,I,L,M,F,P,S,T,W,Y,V;
D18A,G,I,L,M,F,P,W,Y,V;
Q20A,C,I,L,M,F,P,S,T,W,Y,V;
R23A,G,I,L,M,F,P,W,Y,V;
R24A,G,I,L,M,F,P,W,Y,V;
15 Q26A,C,G,I,L,M,F,P,S,T,W,V;
E34A,G,I,L,M,F,P,W,Y,V;
H35A,G,M,F,P,W,Y,V;
T49A,G,P;
S50A,G,M,F,P,W;
20 Q51A,C,G,I,L,M,F,P,S,T,W,Y,V;
A52P;
D53A,G,M,P;
L61I,M,Y;
H68A,G,I,L,M,F,P,W,Y,V;
25 V73M,P,W,Y;
Q84A,C,G,I,L,M,F,P,S,T,W,Y,V;
S85A,G,I,L,M,F,P,T,W,Y,V;
K88A,G,I,L,M,F,P,W,Y,V;
H91A,G,I,L,M,F,P,W,Y,V;
30 S92A,G,I,L,M,F,P,T,W,Y,V;
N96A,C,G,I,L,M,F,P,S,T,W,Y,V;
K106A,G,I,L,M,P,Y,V;
G108I,L,M,F,P,W,Y,V;
D114A,G,F,P,W,Y;
35 T116A,G,I,L,M,F,P,W,Y,V;
E119A,G,I,L,M,F,P,W,Y,V;

- D121A,G,I,L,M,F,P,W,Y,V;
P122I,L,M,F,W,Y;
A123I,L,M,F,P,W,Y,V;
D124G,I,L,M,F,P,W,Y,V;
5 R125G,I,M,F,W,Y;
N126Q,G,I,L,M,F,P,S,T,W,Y,V;
R127A,G,I,L,M,F,P,,W,Y,V;
V128I,L,M,F,P,W,Y;
I129F,W,Y;
10 S130A,G,I,L,M,F,P,W,Y,V;
G131A,I,L,M,F,P,W,Y,V;
E132G,I,L,M,F,W,Y;
H133M,W,V;
L134I,F,W,Y;
15 K136A,G,I,L,M,F,P,W,Y,V;
G145A,I,L,M,P,Y,V;
G147A,I,L,M,P,W,Y,V;
S148A,G,I,L,M,F,P,T,W,Y,V;
T149A,G,L,M,F,P,W,Y,V;
20 Y150W;
D152A,G,I,L,M,F,P,W,Y,V;
F153W;
K154A,G,I,L,M,F,P,W,Y,V;
H156A,G,I,L,M,F,P,W,V;
25 Y158W;
D164I,L,M,F,P,W,Y,V;
E167A,G,I,L,M,F,P,W,Y,V;
S168A,G,I,L,M,F,T,W,V;
R169A,G,M,P,W,Y,V;
30 K170A,G,I,L,M,F,P,W,Y,V;
L171W,Y;
N172A,C,Q,G,I,L,M,F,P,T,W,Y,V;
R173A,G,M,P,W,Y,V;
K176A,G,I,L,M,F,P,W,Y,V;
35 G179I,L,M,F,P,W,Y,V;
K180A,G,I,L,M,F,P,W,Y,V;

E185I,L,M,F,P,W,Y,V;
N188A,Q,G,L,M,F,W,V;
E189A,G,I,L,M,F,P,W,Y,V;
G191A,I,L,M,F,P,W,Y,V;
5 Y193W;
L196I,W;
Y198W;
D204L,M,F,P,W,Y,V;
H205A,G,I,L,M,F,P,W,Y,V;
10 P206G,I,L,M,F,W,Y,V;
A209P,W,Y;
A210G,I,L,M,F,P,W,Y,V;
R214A,G,I,L,M,F,P,Y,V;
T217A,G,I,L,M,F,P,W,Y;
15 N221A,C,Q,G,I,L,M,F,P,S,T,W,Y,V;
E222A,G,I,L,M,F,P,W,Y,V;
K234A,G,I,M,F,P,W,Y,V;
H235A,G,I,L,M,F,P,W,Y,V;
K237A,G,I,L,M,F,W,Y,V;
20 S239G,I,L,M,F,P,T,Y,V;
R242G,I,L,M,F,W,Y,V;
N246A,C,Q,G,I,L,M,F,P,S,T,W,Y,V;
H247G,I,L,M,F,P,W,V;
R249A,G,I,L,M,F,P,W,Y,V;
25 E250A,I,L,M,P,W,Y,V;
K251G,I,L,M,F,P,W,Y,V;
T252A,G,I,L,M,F,P,W,Y,V;
G253I,L,M,F,P,W,Y;
K254A,G,I,L,M,F,P,W,Y,V;
30 E255A,G,I,L,M,F,W,Y,V;
F257W;
E261A,G,I,L,M,F,P,W,Y,V;
N265C,Q,I,L,M,F,P,W;
D266A,G,I,L,M,F,P,W,Y,V;
35 L267W,Y;
G268A,I,L,M,F,P,W,Y,V;

- A269I,L,M,F,P,W,Y,V;
E271A,G,I,L,M,F,P,W,Y,V;
N272A,C,Q,G,I,L,M,F,P,S,T,W,Y,V;
N275A,C,Q,G,I,L,M,F,P,S,W,Y,V;
5 K276A,G,I,L,M,F,P,W,Y,V;
N278A,C,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
N280A,C,G,I,L,M,F,P,W,Y,V;
Y290W;
Q291A,G,I,L,M,F,P,S,T,W,Y,V;
10 H293A,G,I,L,M,P,W,V;
A294I,L,M,F,P,W,Y;
T297A,G,I,L,M,F,P,W,Y,V;
Q298G,I,L,M,F,P,S,T,W,Y,V;
G299A,M,P;
15 G300A,I,L,M,F,P,W,Y,V;
G301I,L,M,F,P,W,Y,V;
Y302W;
D303I,L,M,F,P,W,Y,V;
R305G,I,L,M,F,P,W,Y,V;
20 K306G,I,L,M,F,P,W,Y,V;
L308I,F,W,Y;
N309Q,G,I,L,M,F,P,S,T,W,Y,V;
G310A,I,L,M,F,P,W,Y,V;
S314A,G,I,L,M,F,P,W,Y,V;
25 K315A,G,I,L,M,F,P,W,Y,V;
H316A,G,I,L,M,F,P,W,V;
P317I,L,M,F,W,Y;
L318I,W,Y;
K319A,G,I,L,M,F,P,W,Y,V;
30 D328A,G,I,L,M,F,P,W,Y,V;
G332A,I,L,M,F,P,W,Y,V;
Q333A,C,G,I,M,F,P,S,T,Y,V;
S334G,M,F,P,W,Y;
L335I,F,W,Y;
35 E336A,G,I,L,M,F,P,W,Y,V;
S337A,G,I,L,M,F,P,T,W,Y,V;

- T338A,G,I,L,M,F,P,W,Y,V;
Q340I,L,M,F,P,S,T,W,Y,V;
T341A,G,I,L,M,F,W,Y,V;
P345I,L,M,F,W,Y;
5 E355A,G,I,L,M,F,P,W,Y,V;
Y358W;
Y363W;
K370A,G,I,L,M,F,P,W,Y,V;
G371A,I,L,M,F,P,W,Y,V;
10 S373A,G,I,L,M,F,T,W,Y,V;
Q374A,C,G,I,L,M,F,S,T,W,Y,V;
R375A,G,I,L,M,F,P,W,V;
E376A,G,I,L,M,F,P,W,Y,V;
P378G,I,L,M,F,W,Y,V;
15 A379I,L,M,F,P,W,Y,V;
K381A,G,I,L,M,F,P,W,Y,V;
K389A,G,M,F,P,W,Y,V;
Q393A,C,G,I,L,M,F,P,S,T,W,Y,V;
Y394W;
20 Y396W;
A398I,L,M,F,W,Y,V;
Y402W;
F403W;
D404I,L,M,F,P,W,Y,V;
25 H405G,I,L,M,F,P,W,Y,V;
H406A,G,I,M,F,P,Y,V;
D407A,G,I,L,M,F,P,W,Y,V;
I408W,Y;
R413A,G,I,L,M,F,P,W,Y,V;
30 E414A,G,I,L,M,F,P,W,Y,V;
G415A,I,L,M,F,P,W,Y,V;
D416A,G,I,L,M,F,P,W,Y,V;
S417A,G,I,L,M,F,P,W,Y,V;
S418A,G,I,L,M,F,P,T,W,Y,V;
35 V419I,L,M,F,P,W,Y;
A420I,L,M,F,W,Y,V;

- N421A,C,Q,I,L,M,F,P,S,T,W,Y,V;
S422A,G,I,L,M,F,P,T,W,Y,V;
G431A,I,L,M,F,P,W,Y,V;
P432I,L,M,F,W,Y;
5 G433A,I,L,M,F,P,W,Y,V;
G434A,I,L,M,F,P,W,Y,V;
A435I,L,M,F,P,W,Y,V;
K436A,G,I,L,M,F,P,W,Y,V;
R437A,G,I,L,M,F,P,W,Y,V;
10 Y439W;
R442A,G,I,L,M,F,P,W,Y,V;
Q443A,C,G,I,L,M,F,P,S,T,W,Y,V;
N444A,C,G,I,L,M,F,P,S,T,W,Y,V;
A445I,L,M,F,P,W,Y,V;
15 G446A,I,L,M,F,P,W,Y,V;
E447A,G,I,L,M,F,P,W,Y,V;
T448A,G,I,L,M,F,P,W,Y,V;
H450A,G,I,L,M,F,P,W,V;
G454A,I,L,M,F,P,W,Y,V;
20 N455A,C,Q,G,I,L,M,F,P,S,T,W,Y,V;
R456A,I,L,M,F,P,W,Y,V;
S457A,G,I,L,M,F,W,Y,V;
E458A,G,I,L,M,F,P,W,Y,V;
P459I,L,M,F,W,Y;
25 V460I,L,M,F,P,W,Y;
V461I,L,M,F,P,W,Y;
N463A,C,Q,I,L,M,F,P,S,T,W,Y,V;
S464A,G,I,L,M,F,P,T,W,Y,V;
E465A,G,I,L,M,F,P,W,Y,V;
30 E469A,G,I,L,M,F,P,W,Y,V;
N473Q,G,I,L,M,F,P,S,T,W,Y,V;
G474A,I,L,M,F,P,W,Y,V;
G475A,I,L,M,F,P,W,Y,V;
S476G,I,L,M,F,P,T,W,Y,V;
35 Q482A,C,G,I,L,M,F,S,T,W,Y,V;
R483A,G,I,L,M,F,P,W,Y,V.

General mutations in variants of the invention

A variant of the invention may in one embodiment comprise one or more modifications in addition to those outlined above. Thus, it may be advantageous that one or more Proline (Pro) residues present in the part of the alpha-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, Valine or Leucine.

Analogously, in one embodiment one or more Cysteine residues present in the parent alpha-amylase may be replaced with a non-Cysteine residue such as Serine, Alanine, Threonine, Glycine, Valine or 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 the amino acid fragment 185-209 of SEQ ID NO: 10 is replaced by an Asn and/or Gln, respectively. Also of interest is the replacement, in the Termamyl-like alpha-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: 10 by an Arg.

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

Furthermore, it may be advantageous to introduce mutations in one or more of the following positions (using SEQ ID NO: 8 (Termamyl) for the numbering):

M15, V128, A111, H133, W138, T149, M197, N188, A209, A210, H405, T412, in particular the following single, double or triple or multi mutations:

M15X, in particular M15T,L;

V128X, in particular V128E;

H133X, in particular H133Y;

N188X, in particular N188S,T,P;

M197X, in particular M197T,L;

A209X, in particular A209V;

M197T/W138F; M197T/W138Y; M15T/H133Y/N188S;

M15/V128E/H133Y/N188S; E119C/S130C; D124C/R127C; H133Y/T149I;

G475R, H133Y/S187D; H133Y/A209V.

Methods for preparing alpha-amylase variants of the invention

Several methods for introducing mutations into genes are known in the art. After a brief description of cloning of alpha-amylase-encoding DNA sequences, methods for generating

mutations at specific sites within the alpha-amylase-encoding sequence will be described.

Cloning a DNA sequence encoding an alpha-amylase

The DNA sequence encoding a parent alpha-amylase may be isolated from any cell or
5 microorganism producing the alpha-amylase in 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 alpha-amylase to be studied.
Then, if the amino acid sequence of the alpha-amylase is known, homologous, labeled oligonu-
cleotide probes may be synthesized and used to identify alpha-amylase-encoding clones from a
10 genomic library prepared from the organism in question. Alternatively, a labeled oligonucleotide
probe containing sequences homologous to a known alpha-amylase gene could be used as a
probe to identify alpha-amylase-encoding clones, using hybridization and washing conditions of
lower stringency.

Yet another method for identifying alpha-amylase-encoding clones would involve inserting
15 fragments of genomic DNA into an expression vector, such as a plasmid, transforming alpha-
amylase-negative bacteria with the resulting genomic DNA library, and then plating the
transformed bacteria onto agar containing a substrate for alpha-amylase, thereby allowing
clones expressing the alpha-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, Tetrahedron Letters **22**, 1981, pp. 1859-1869, or the method described by Matthes et al., The EMBO J. **3**, 1984, pp. 801-805. 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., Science **239**, 1988, pp. 487-491.

Site-directed mutagenesis

Once an alpha-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 alpha-amylase-encoding sequence, is created in a vector carrying the alpha-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 disclose 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.

Another method for introducing mutations into alpha-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. 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.

Alternative methods for providing variants of the invention include gene shuffling, e.g., as described in WO 95/22625 (from Affymax Technologies N.V.) or in WO 96/00343 (from Novo Nordisk A/S), or other corresponding techniques resulting in a hybrid enzyme comprising the mutation(s), e.g., substitution(s) and/or deletion(s), in question.

5

Expression of alpha-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, 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 encoding an alpha-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 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 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 suitable promoters for directing the transcription of the DNA sequence encoding an alpha-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* alpha-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* alpha-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 the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral alpha-amylase, *A. niger* acid stable alpha-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

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 alpha-amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same 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
5 pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

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. Furthermore, the vector may comprise *Aspergillus* selection markers such
10 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* alpha-amylases mentioned herein comprise a preregion permitting
15 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 alpha-amylase variant, the promoter, terminator and other elements, respectively, and to insert
20 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
25 of an alpha-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
30 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.

35 Examples of suitable bacteria are 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*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram-negative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

5 The yeast organism may favorably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces 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 followed by regeneration of the cell wall in a manner known *per se*. A suitable
10 procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

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

15 The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the alpha-amylase variant of the invention. 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).

20 The alpha-amylase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known 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,
25 or the like.

INDUSTRIAL APPLICATIONS

The alpha-amylase variants of this invention possess valuable properties allowing for a variety of industrial applications. In particular, enzyme variants of the invention are applicable as
30 a component in washing, dishwashing, and hard surface cleaning detergent compositions.

Variant of the invention with altered properties may be used for starch processes, in particular starch conversion, especially liquefaction of starch (see, e.g., US 3,912,590, EP patent application nos. 252 730 and 63 909, WO 99/19467, and WO 96/28567 all references hereby incorporated by reference). Also contemplated are compositions for starch conversion
35 purposes, which may beside the variant of the invention also comprise a glucoamylase, pullulanase, and other alpha-amylases.

Further, variants of the invention are also particularly useful in the production of sweeteners and ethanol (see, e.g., US patent no. 5,231,017 hereby incorporated by reference), such as fuel, drinking and industrial ethanol, from starch or whole grains.

5 Variants of the invention may also be useful for desizing of textiles, fabrics and garments (see, e.g., WO 95/21247, US patent 4,643,736, EP 119,920 hereby incorporated by reference), beer making or brewing, in pulp and paper production.

Starch Conversion

10 Conventional starch-conversion processes, such as liquefaction and saccharification processes are described, e.g., in US Patent No. 3,912,590 and EP patent publications Nos. 252,730 and 63,909, hereby incorporated by reference.

In an embodiment the starch conversion process degrading starch to lower molecular weight carbohydrate components such as sugars or fat replacers includes a debranching step.

15

Starch to sugar conversion

In the case of converting starch into a sugar the starch is depolymerized. A such depolymerization process consists of a Pre-treatment step and two or three consecutive process steps, viz. a liquefaction process, a saccharification process and dependent on the
20 desired end product optionally an isomerization process.

Pre-treatment of native starch

Native starch consists of microscopic granules, which are insoluble in water at room temperature. When an aqueous starch slurry is heated, the granules swell and eventually
25 burst, dispersing the starch molecules into the solution. During this "gelatinization" process there is a dramatic increase in viscosity. As the solids level is 30-40% in a typically industrial process, the starch has to be thinned or "liquefied" so that it can be handled. This reduction in viscosity is today mostly obtained by enzymatic degradation.

30 Liquefaction

During the liquefaction step, the long chained starch is degraded into branched and linear shorter units (maltodextrins)
by an alpha-amylase. The liquefaction process is carried out at 105-110°C for 5 to 10 minutes followed by 1-2 hours at 95°C. The pH lies between 5.5 and 6.2. In order to ensure optimal
35 enzyme stability under these conditions, 1 mM of calcium is added (40 ppm free calcium ions). After this treatment the liquefied starch will have a "dextrose equivalent" (DE) of 10-15.

Saccharification

After the liquefaction process the maltodextrins are converted into dextrose by addition of a glucoamylase (e.g., AMG) and a debranching enzyme, such as an isoamylase (US patent no. 4,335,208) or a pullulanase (e.g., Promozyme™) (US patent no. 4,560,651). Before this step the pH is reduced to a value below 4.5, maintaining the high temperature (above 95°C) to inactivate the liquefying alpha-amylase to reduce the formation of short oligosaccharide called "panose precursors" which cannot be hydrolyzed properly by the debranching enzyme.

The temperature is lowered to 60°C, and glucoamylase and debranching enzyme are added. The saccharification process proceeds for 24-72 hours.

Normally, when denaturing the α -amylase after the liquefaction step about 0.2-0.5% of the saccharification product is the branched trisaccharide 6²-alpha-glucosyl maltose (panose) which cannot be degraded by a pullulanase. If active amylase from the liquefaction step is present during saccharification (i.e., no denaturing), this level can be as high as 1-2%, which is highly undesirable as it lowers the saccharification yield significantly.

Isomerization

When the desired final sugar product is, e.g., high fructose syrup the dextrose syrup may be converted into fructose. 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 glucoseisomerase (such as Sweetzyme™ IT).

Ethanol production

In general alcohol production (ethanol) from whole grain can be separated into 4 main steps

- Milling
- Liquefaction
- Saccharification
- Fermentation

Milling

The grain is milled in order to open up the structure and allowing for further processing. Two processes are used wet or dry milling. In dry milling the whole kernel is

milled and used in the remaining part of the process. Wet milling gives a very good separation of germ and meal (starch granules and protein) and is with a few exceptions applied at locations where there is a parallel production of syrups.

5 Liquefaction

In the liquefaction process the starch granules are solubilized by hydrolysis to maltodextrins mostly of a DP higher than 4. The hydrolysis may be carried out by acid treatment or enzymatically by alpha-amylase. Acid hydrolysis is used on a limited basis. The raw material can be milled whole grain or a side stream from starch processing.

10 Enzymatic liquefaction is typically carried out as a three-step hot slurry process. The slurry is heated to between 60-95°C, preferably 80-85°C, and the enzyme(s) is (are) added. Then the slurry is jet-cooked at between 95-140°C, preferably 105-125°C, cooled to 60-95°C and more enzyme(s) is (are) added to obtain the final hydrolysis. The liquefaction process is carried out at pH 4.5-6.5, typically at a pH between 5 and 6. Milled and liquefied grain is also
15 known as mash.

Saccharification

To produce low molecular sugars DP₁₋₃ that can be metabolized by yeast, the maltodextrin from the liquefaction must be further hydrolyzed. The hydrolysis is typically done
20 enzymatically by glucoamylases, alternatively alpha-glucosidases or acid alpha-amylases can be used. A full saccharification step may last up to 72 hours, however, it is common only to do a pre-saccharification of typically 40-90 minutes and then complete saccharification during fermentation (SSF). Saccharification is typically carried out at temperatures from 30-65°C, typically around 60°C, and at pH 4.5.

25

Fermentation

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

30 Note that the most widely used process is a simultaneous saccharification and fermentation (SSF) process where there is no holding stage for the saccharification, meaning that yeast and enzyme is added together. When doing SSF it is common to introduce a pre-saccharification step at a temperature above 50°C, just prior to the fermentation.

35 Distillation

Following the fermentation the mash is distilled to extract the ethanol.

The ethanol obtained according to the process of the invention may be used as, e.g., fuel ethanol; drinking ethanol, i.e., potable neutral spirits; or industrial ethanol.

By-products

5 Left over from the fermentation is the grain, which is typically used for animal feed either in liquid form or dried.

Further details on how to carry out liquefaction, saccharification, fermentation, distillation, and recovering of ethanol are well known to the skilled person.

According to the process of the invention the saccharification and fermentation may be
10 carried out simultaneously or separately.

Pulp and Paper Production

The alkaline alpha-amylase of the invention may also be used in the production of lignocellulosic materials, such as pulp, paper and cardboard, from starch reinforced waste
15 paper and cardboard, especially where re-pulping occurs at pH above 7 and where amylases facilitate the disintegration of the waste material through degradation of the reinforcing starch. The alpha-amylase of the invention is especially useful in a process for producing a papermaking pulp from starch-coated printed-paper. The process may be performed as described in WO 95/14807, comprising the following steps:

- 20 a) disintegrating the paper to produce a pulp,
b) treating with a starch-degrading enzyme before, during or after step a), and
c) separating ink particles from the pulp after steps a) and b).

The alpha-amylases of the invention may also be very useful in modifying starch where enzymatically modified starch is used in papermaking together with alkaline fillers such
25 as calcium carbonate, kaolin and clays. With the alkaline alpha-amylases of the invention it becomes possible to modify the starch in the presence of the filler thus allowing for a simpler integrated process.

Desizing of Textiles, Fabrics and Garments

30 An alpha-amylase of the invention may also be very useful in textile, fabric or garment desizing. In the textile processing industry, alpha-amylases are traditionally used as auxiliaries in the desizing process to facilitate the removal of starch-containing size, which has served as a protective coating on weft yarns during weaving. Complete removal of the size coating after weaving is important to ensure optimum results in the subsequent processes, in which the
35 fabric is scoured, bleached and dyed. Enzymatic starch breakdown is preferred because it does not involve any harmful effect on the fiber material. In order to reduce processing cost

and increase mill throughput, the desizing processing is sometimes combined with the scouring and bleaching steps. In such cases, non-enzymatic auxiliaries such as alkali or oxidation agents are typically used to break down the starch, because traditional alpha-amylases are not very compatible with high pH levels and bleaching agents. The non-enzymatic breakdown of the starch size does lead to some fiber damage because of the rather aggressive chemicals used. Accordingly, it would be desirable to use the alpha-amylases of the invention as they have an improved performance in alkaline solutions. The alpha-amylases may be used alone or in combination with a cellulase when desizing cellulose-containing fabric or textile.

Desizing and bleaching processes are well known in the art. For instance, such processes are described in WO 95/21247, US patent 4,643,736, EP 119,920 hereby incorporated by reference.

Commercially available products for desizing include AQUAZYME® and AQUAZYME® ULTRA from Novozymes A/S.

Beer making

The alpha-amylases of the invention may also be very useful in a beer-making process; the alpha-amylases will typically be added during the mashing process.

Detergent Compositions

The alpha-amylase of the invention may be added to and thus become a component of a detergent composition.

The detergent composition of the invention may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations, or be formulated for hand or machine dishwashing operations.

In a specific aspect, the invention provides a detergent additive comprising the enzyme of the invention. The detergent additive as well as the detergent composition may comprise one or more other enzymes such as a protease, a lipase, a peroxidase, another amylolytic enzyme, e.g., another alpha-amylase, glucoamylase, maltogenic amylase, CGTase and/or a cellulase, mannanase (such as MANNAWAY™ from Novozymes, Denmark)), pectinase, pectine lyase, cutinase, and/or laccase.

In general the properties of the chosen enzyme(s) should be compatible with the selected detergent, (i.e., pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.), and the enzyme(s) should be present in effective amounts.

Proteases: Suitable proteases include those of animal, vegetable or microbial origin.

Microbial origin is preferred. Chemically modified or protein engineered mutants are included. The protease may be a serine protease or a metallo protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g., of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270 and WO 94/25583.

Examples of useful proteases are the variants described in WO 92/19729, WO 98/20115, WO 98/20116, and WO 98/34946, especially the variants with substitutions in one or more of the following positions: 27, 36, 57, 76, 87, 97, 101, 104, 120, 123, 167, 170, 194, 206, 218, 222, 224, 235 and 274.

Preferred commercially available protease enzymes include ALCALASE®, SAVINASE®, PRIMASE®, DURALASE®, ESPERASE®, and KANNASE® (from Novozymes A/S), MAXATASE®, MAXACAL, MAXAPEM®, PROPERASE®, PURAFECT®, PURAFECT OXP®, FN2®, FN3®, FN4® (Genencor International Inc.).

Lipases: Suitable lipases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful lipases include lipases from *Humicola* (synonym *Thermomyces*), e.g., from *H. lanuginosa* (*T. lanuginosus*) as described in EP 258 068 and EP 305 216 or from *H. insolens* as described in WO 96/13580, a *Pseudomonas* lipase, e.g., from *P. alcaligenes* or *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *P. stutzeri* (GB 1,372,034), *P. fluorescens*, *Pseudomonas* sp. strain SD 705 (WO 95/06720 and WO 96/27002), *P. wisconsinensis* (WO 96/12012), a *Bacillus* lipase, e.g., from *B. subtilis* (Dartois et al. (1993), Biochemica et Biophysica Acta, 1131, 253-360), *B. stearothermophilus* (JP 64/744992) or *B. pumilus* (WO 91/16422).

Other examples are lipase variants such as those described in WO 92/05249, WO 94/01541, EP 407 225, EP 260 105, WO 95/35381, WO 96/00292, WO 95/30744, WO 94/25578, WO 95/14783, WO 95/22615, WO 97/04079 and WO 97/07202.

Preferred commercially available lipase enzymes include LIPOLASE™ and LIPOLASE ULTRA™ (Novozymes A/S).

Amylases: Suitable amylases (alpha and/or beta) include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Amylases include, for example, alpha-amylases obtained from *Bacillus*, e.g., a special strain of *B. licheniformis*, described in more detail in GB 1,296,839. Examples of useful alpha-amylases are the variants described in WO 94/02597, WO 94/18314, WO 96/23873, and WO 97/43424, especially the variants with substitutions in one or more of the following positions: 15, 23, 105, 106, 124, 128, 133, 154, 156, 181, 188, 190, 197, 202, 208, 209, 243, 264, 304, 305, 391, 408, and

444.

Commercially available alpha-amylases are DURAMYL™, LIQUEZYME™, TERMAMYL™, NATALASE™, FUNGAMYL™ and BAN™ (Novozymes A/S), RAPIDASE™ and PURASTAR™ (from Genencor International Inc.).

5 Cellulases: Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera *Bacillus*, *Pseudomonas*, *Humicola*, *Fusarium*, *Thielavia*, *Acremonium*, e.g., the fungal cellulases produced from *Humicola insolens*, *Myceliophthora thermophila* and *Fusarium oxysporum* disclosed in US 4,435,307, US 5,648,263, US 5,691,178, US 5,776,757
10 and WO 89/09259.

Especially suitable cellulases are the alkaline or neutral cellulases having colour care benefits. Examples of such cellulases are cellulases described in EP 0 495 257, EP 0 531 372, WO 96/11262, WO 96/29397, WO 98/08940. Other examples are cellulase variants such as those described in WO 94/07998, EP 0 531 315, US 5,457,046, US 5,686,593, US
15 5,763,254, WO 95/24471, WO 98/12307 and PCT/DK98/00299.

Commercially available cellulases include CELLUZYME®, and CAREZYME® (Novozymes A/S), CLAZINASE®, and PURADAX HA® (Genencor International Inc.), and KAC-500(B)® (Kao Corporation).

Peroxidases/Oxidases: Suitable peroxidases/oxidases include those of plant, bac-
20 terial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from *Coprinus*, e.g., from *C. cinereus*, and variants thereof as those described in WO 93/24618, WO 95/10602, and WO 98/15257.

Commercially available peroxidases include GUARDZYME® (Novozymes A/S).

The detergent enzyme(s) may be included in a detergent composition by adding
25 separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, i.e., a separate additive or a combined additive, can be formulated, e.g., granulate, a liquid, a slurry, etc. Preferred detergent additive formulations are granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids, or slurries.

Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000; ethoxylated nonyl-phenols having from 16 to 50 ethylene oxide units; 5 ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591. Liquid enzyme pre-parations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or 10 boric acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g., a bar, a tablet, a powder, a granule, a paste or a liquid. A liquid detergent may be aqueous, typically containing up to 70 % water and 0-30 % organic solvent, or non-aqueous.

15 The detergent composition comprises one or more surfactants, which may be non-ionic including semi-polar and/or anionic and/or cationic and/or zwitterionic. The surfactants are typically present at a level of from 0.1% to 60% by weight.

When included therein the detergent will usually contain from about 1% to about 40% of an anionic surfactant such as linear alkylbenzenesulfonate, alpha-olefinsulfonate, alkyl sulfate (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, alpha-sulfo fatty acid 20 methyl ester, alkyl- or alkenylsuccinic acid or soap.

When included therein the detergent will usually contain from about 0.2% to about 40% of a non-ionic surfactant such as alcohol ethoxylate, nonyl-phenol ethoxylate, alkylpolyglycoside, alkyldimethylamine-oxide, ethoxylated fatty acid monoethanol-amide, fatty 25 acid monoethanolamide, polyhydroxy alkyl fatty acid amide, or N-acyl N-alkyl derivatives of glucosamine ("glucamides").

The detergent may contain 0-65 % of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, carbonate, citrate, nitrilotriacetic acid, ethylenediaminetetraacetic acid, diethylenetri-aminepentaacetic acid, alkyl- or alkenylsuccinic 30 acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst).

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose, poly(vinyl-pyrrolidone), poly(ethylene glycol), poly(vinyl alcohol), poly(vinylpyridine-N-oxide), poly(vinylimidazole), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid co-polymers.

35 The detergent may contain a bleaching system, which may comprise a H₂O₂ source such as perborate or percarbonate which may be combined with a peracid-forming bleach

activator such as tetraacetythylenediamine or nonanoyloxybenzenesulfonate. Alternatively, the bleaching system may comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

The enzyme(s) of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g., a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g., an aromatic borate ester, or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as e.g. fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil re-deposition agents, dyes, bactericides, optical brighteners, hydrotropes, tarnish inhibitors, or perfumes.

It is at present contemplated that in the detergent compositions any enzyme, in particular the enzyme of the invention, may be added in an amount corresponding to 0.01-100 mg of enzyme protein per liter of wash liquor, preferably 0.05-5 mg of enzyme protein per liter of wash liquor, in particular 0.1-1 mg of enzyme protein per liter of wash liquor.

The enzyme of the invention may additionally be incorporated in the detergent formulations disclosed in WO 97/07202, which is hereby incorporated as reference.

Dishwash Detergent Compositions

The enzyme of the invention may also be used in dish wash detergent compositions, including the following:

1) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	0.4 - 2.5%
Sodium metasilicate	0 - 20%
Sodium disilicate	3 - 20%
Sodium triphosphate	20 - 40%
Sodium carbonate	0 - 20%
Sodium perborate	2 - 9%
Tetraacetyl ethylene diamine (TAED)	1 - 4%
Sodium sulphate	5 - 33%
Enzymes	0.0001 - 0.1%

2) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant (e.g. alcohol ethoxylate)	1 - 2%
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Sodium disilicate	2 - 30%
Sodium carbonate	10 - 50%
Sodium phosphonate	0 - 5%
Trisodium citrate dihydrate	9 - 30%
Nitrilotrisodium acetate (NTA)	0 - 20%
Sodium perborate monohydrate	5 - 10%
Tetraacetyl ethylene diamine (TAED)	1 - 2%
Polyacrylate polymer (e.g. maleic acid/acrylic acid copolymer)	6 - 25%
Enzymes	0.0001 - 0.1%
Perfume	0.1 - 0.5%
Water	5 - 10

3) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	0.5 - 2.0%
Sodium disilicate	25 - 40%
Sodium citrate	30 - 55%
Sodium carbonate	0 - 29%
Sodium bicarbonate	0 - 20%
Sodium perborate monohydrate	0 - 15%
Tetraacetyl ethylene diamine (TAED)	0 - 6%
Maleic acid/acrylic acid copolymer	0 - 5%
Clay	1 - 3%
Polyamino acids	0 - 20%
Sodium polyacrylate	0 - 8%
Enzymes	0.0001 - 0.1%

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4) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	1 - 2%
Zeolite MAP	15 - 42%
Sodium disilicate	30 - 34%
Sodium citrate	0 - 12%

Sodium carbonate	0 - 20%
Sodium perborate monohydrate	7 - 15%
Tetraacetyl ethylene diamine (TAED)	0 - 3%
Polymer	0 - 4%
Maleic acid/acrylic acid copolymer	0 - 5%
Organic phosphonate	0 - 4%
Clay	1 - 2%
Enzymes	0.0001 - 0.1%
Sodium sulphate	Balance

5) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	1 - 7%
Sodium disilicate	18 - 30%
Trisodium citrate	10 - 24%
Sodium carbonate	12 - 20%
Monopersulphate (2 KHSO ₅ .KHSO ₄ .K ₂ SO ₄)	15 - 21%
Bleach stabilizer	0.1 - 2%
Maleic acid/acrylic acid copolymer	0 - 6%
Diethylene triamine pentaacetate, pentasodium salt	0 - 2.5%
Enzymes	0.0001 - 0.1%
Sodium sulphate, water	Balance

5 6) POWDER AND LIQUID DISHWASHING COMPOSITION WITH CLEANING SURFACTANT SYSTEM

Nonionic surfactant	0 - 1.5%
Octadecyl dimethylamine N-oxide dihydrate	0 - 5%
80:20 wt.C18/C16 blend of octadecyl dimethylamine N-oxide dihydrate and hexadecyldimethyl amine N-oxide dihydrate	0 - 4%
70:30 wt.C18/C16 blend of octadecyl bis (hydroxyethyl)amine N-oxide anhydrous and hexadecyl bis (hydroxyethyl)amine N-oxide anhydrous	0 - 5%

C ₁₃ -C ₁₅ alkyl ethoxysulfate with an average degree of ethoxylation of 3	0 - 10%
C ₁₂ -C ₁₅ alkyl ethoxysulfate with an average degree of ethoxylation of 3	0 - 5%
C ₁₃ -C ₁₅ ethoxylated alcohol with an average degree of ethoxylation of 12	0 - 5%
A blend of C ₁₂ -C ₁₅ ethoxylated alcohols with an average degree of ethoxylation of 9	0 - 6.5%
A blend of C ₁₃ -C ₁₅ ethoxylated alcohols with an average degree of ethoxylation of 30	0 - 4%
Sodium disilicate	0 - 33%
Sodium tripolyphosphate	0 - 46%
Sodium citrate	0 - 28%
Citric acid	0 - 29%
Sodium carbonate	0 - 20%
Sodium perborate monohydrate	0 - 11.5%
Tetraacetyl ethylene diamine (TAED)	0 - 4%
Maleic acid/acrylic acid copolymer	0 - 7.5%
Sodium sulphate	0 - 12.5%
Enzymes	0.0001 - 0.1%

7) NON-AQUEOUS LIQUID AUTOMATIC DISHWASHING COMPOSITION

Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0 - 10.0%
Alkali metal silicate	3.0 - 15.0%
Alkali metal phosphate	20.0 - 40.0%
Liquid carrier selected from higher glycols, polyglycols, polyoxides, glycolethers	25.0 - 45.0%
Stabilizer (e.g. a partial ester of phosphoric acid and a C ₁₆ -C ₁₈ alkanol)	0.5 - 7.0%
Foam suppressor (e.g. silicone)	0 - 1.5%
Enzymes	0.0001 - 0.1%

5 8) NON-AQUEOUS LIQUID DISHWASHING COMPOSITION

Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0 - 10.0%
Sodium silicate	3.0 - 15.0%

Alkali metal carbonate	7.0 - 20.0%
Sodium citrate	0.0 - 1.5%
Stabilizing system (e.g. mixtures of finely divided silicone and low molecular weight dialkyl polyglycol ethers)	0.5 - 7.0%
Low molecule weight polyacrylate polymer	5.0 - 15.0%
Clay gel thickener (e.g. bentonite)	0.0 - 10.0%
Hydroxypropyl cellulose polymer	0.0 - 0.6%
Enzymes	0.0001 - 0.1%
Liquid carrier selected from higher lycols, polyglycols, polyoxides and glycol ethers	Balance

9) THIXOTROPIC LIQUID AUTOMATIC DISHWASHING COMPOSITION

C ₁₂ -C ₁₄ fatty acid	0 - 0.5%
Block co-polymer surfactant	1.5 - 15.0%
Sodium citrate	0 - 12%
Sodium tripolyphosphate	0 - 15%
Sodium carbonate	0 - 8%
Aluminium tristearate	0 - 0.1%
Sodium cumene sulphonate	0 - 1.7%
Polyacrylate thickener	1.32 - 2.5%
Sodium polyacrylate	2.4 - 6.0%
Boric acid	0 - 4.0%
Sodium formate	0 - 0.45%
Calcium formate	0 - 0.2%
Sodium n-decydiphenyl oxide disulphonate	0 - 4.0%
Monoethanol amine (MEA)	0 - 1.86%
Sodium hydroxide (50%)	1.9 - 9.3%
1,2-Propanediol	0 - 9.4%
Enzymes	0.0001 - 0.1%
Suds suppressor, dye, perfumes, water	Balance

10) LIQUID AUTOMATIC DISHWASHING COMPOSITION

Alcohol ethoxylate	0 - 20%
Fatty acid ester sulphonate	0 - 30%
Sodium dodecyl sulphate	0 - 20%
Alkyl polyglycoside	0 - 21%
Oleic acid	0 - 10%
Sodium disilicate monohydrate	18 - 33%
Sodium citrate dihydrate	18 - 33%
Sodium stearate	0 - 2.5%
Sodium perborate monohydrate	0 - 13%
Tetraacetyl ethylene diamine (TAED)	0 - 8%
Maleic acid/acrylic acid copolymer	4 - 8%
Enzymes	0.0001 - 0.1%

11) LIQUID AUTOMATIC DISHWASHING COMPOSITION CONTAINING PROTECTED BLEACH PARTICLES

Sodium silicate	5 - 10%
Tetrapotassium pyrophosphate	15 - 25%
Sodium triphosphate	0 - 2%
Potassium carbonate	4 - 8%
Protected bleach particles, e.g. chlorine	5 - 10%
Polymeric thickener	0.7 - 1.5%
Potassium hydroxide	0 - 2%
Enzymes	0.0001 - 0.1%
Water	Balance

5 11) Automatic dishwashing compositions as described in 1), 2), 3), 4), 6) and 10), wherein perborate is replaced by percarbonate.

12) Automatic dishwashing compositions as described in 1) - 6) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", *Nature* **369**, 1994, pp. 637-639.

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Uses

The present invention is also directed to methods for using an alpha-amylase variant of the invention in detergents, in particular laundry detergent compositions and dishwashing

detergent compositions, hard surface cleaning compositions, and in composition for desizing of textiles, fabrics or garments, for production of pulp and paper, beer making, ethanol production, and starch conversion processes as described above.

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Compositions

The invention also related to composition comprising a variant of the invention, and in a preferred embodiment also a *B. stearrowthermophilus* alpha-amylase (BSG), in particular a variant thereof.

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In another embodiment the composition comprises beside a variant of the invention a glucoamylase, in particular a glucoamylase originating from *Aspergillus niger* (e.g., the G1 or G2 *A. niger* AMG disclosed in Boel et al. (1984), "Glucoamylases G1 and G2 from *Aspergillus niger* are synthesized from two different but closely related mRNAs", EMBO J. 3 (5), p. 1097-1102, or a variant therefore, in particular a variant disclosed in WO 00/04136 or WO 01/04273

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or the *Talaromyces emersonii* AMG disclosed in WO 99/28448.

In an embodiment the composition of the invention also comprises a pullulanase, in particular a *Bacillus* pullulanase.

MATERIALS AND METHODS

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Materials:

Bacillus licheniformis alpha-amylase shown in SEQ ID NO: 8 and also available from Novozymes A/S, Denmark.

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AA560: SEQ ID NO: 12; disclosed in WO 00/60060; deposited on 25th January 1999 at DSMZ and assigned the DSMZ no. 12649.

LB medium (In 1 liter H₂O: 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl, pH adjusted to 7.0 w. NaOH, autoclaved).

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TY agar plates (In 1 liter H₂O: 16 g bacto-tryptone, 10 g bacto-yeast extract, 5 g NaCl, pH adjusted to 7.0 w. NaOH, and 15 g bacto-agar is added prior to autoclaving).

10% Lugol solution (Iodine/Potassium iodine solution; made by 10-fold dil. in H₂O of stock: Sigma Cat. no. L 6146).

Bacillus subtilis SHA273: see WO 95/10603

Detergents:

Model detergent: A/P (Asia/Pacific) Model Detergent has the following composition: 20% STPP (sodium tripolyphosphate), 25% Na₂SO₄, 15% Na₂CO₃, 20% LAS (linear alkylbenzene sulfonate, Nansa 80S), 5% C12-C15 alcohol ethoxylate (Dobanol 25-7), 5% Na₂SiO₅, 0.3% NaCl.

Omo Multi Acao (Brazil),

Omo concentrated powder (EU) (Unilever)

10 Ariel Futur liquid (EU) (Procter and Gamble)

Ariel Essential (EU) (Procter and Gamble)

Plasmids

pDN1528 contains the complete gene encoding Termamyl, *amyL*, the expression of which is directed by its own promoter. Further, the plasmid contains the origin of replication, *ori*, from plasmid pUB110 and the *cat* gene from plasmid pC194 conferring resistance towards chloramphenicol. pDN1528 is shown in Fig. 9 of WO 96/23874.

Methods:

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Filter screening assays

The below assays can be used to screening of Termamyl-like alpha-amylase variants having altered stability at high or low pH and/or under Ca²⁺ depleted conditions compared to the parent enzyme and Termamyl-like alpha-amylase.

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High pH filter assay

Bacillus libraries are plated on a sandwich of cellulose acetate (OE 67, Schleicher & Schuell, Dassel, Germany) - and nitrocellulose filters (Protran-Ba 85, Schleicher & Schuell, Dassel, Germany) on TY agar plates with 10 micro g/ml kanamycin at 37°C for at least 21 hours. The cellulose acetate layer is located on the TY agar plate.

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Each filter sandwich is specifically marked with a needle after plating, but before incubation in order to be able to localize positive variants on the filter and the nitrocellulose filter with bound variants is transferred to a container with glycine-NaOH buffer, pH 8.6-10.6 and incubated at room temperature (can be altered from 10-60°C) for 15 min. The cellulose acetate filters with colonies are stored on the TY-plates at room temperature until use. After incubation, residual activity is detected on plates containing 1% agarose, 0.2% starch in

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glycin-NaOH buffer, pH 8.6-10.6. The assay plates with nitrocellulose filters are marked the same way as the filter sandwich and incubated for 2 hours at room temperature. After removal of the filters the assay plates are stained with 10% Lugol solution. Starch degrading variants are detected as white spots on dark blue background and then identified on the storage plates. Positive variants are rescreened twice under the same conditions as the first screen.

Low calcium filter assay

Bacillus libraries are plated on a sandwich of cellulose acetate (OE 67, Schleicher & Schuell, Dassel, Germany) - and nitrocellulose filters (Protran-Ba 85, Schleicher & Schuell, Dassel, Germany) on TY agar plates with a relevant antibiotic, e.g., kanamycin or chloramphenicol, at 37°C for at least 21 hours. The cellulose acetate layer is located on the TY agar plate.

Each filter sandwich is specifically marked with a needle after plating, but before incubation in order to be able to localize positive variants on the filter and the nitrocellulose filter with bound variants is transferred to a container with carbonate/bicarbonate buffer pH 8.5-10 and with different EDTA concentrations (0.001 mM - 100 mM). The filters are incubated at room temperature for 1 hour. The cellulose acetate filters with colonies are stored on the TY-plates at room temperature until use. After incubation, residual activity is detected on plates containing 1% agarose, 0.2% starch in carbonate/bicarbonate buffer pH 8.5-10. The assay plates with nitrocellulose filters are marked the same way as the filter sandwich and incubated for 2 hours at room temperature. After removal of the filters the assay plates are stained with 10% Lugol solution. Starch degrading variants are detected as white spots on dark blue background and then identified on the storage plates. Positive variants are rescreened twice under the same conditions as the first screen.

Low pH filter assay

Bacillus libraries are plated on a sandwich of cellulose acetate (OE 67, Schleicher & Schuell, Dassel, Germany) - and nitrocellulose filters (Protran-Ba 85, Schleicher & Schuell, Dassel, Germany) on TY agar plates with 10 micro g/ml chloramphenicol at 37°C for at least 21 hours. The cellulose acetate layer is located on the TY agar plate.

Each filter sandwich is specifically marked with a needle after plating, but before incubation in order to be able to localize positive variants on the filter, and the nitrocellulose filter with bound variants is transferred to a container with citrate buffer, pH 4.5 and incubated at 80°C for 20 minutes (when screening for variants in the wild type backbone) or 85°C for 60 minutes (when screening for variants of the parent alpha-amylase). The cellulose

acetate filters with colonies are stored on the TY-plates at room temperature until use. After incubation, residual activity is detected on assay plates containing 1% agarose, 0.2% starch in citrate buffer, pH 6.0. The assay plates with nitrocellulose filters are marked the same way as the filter sandwich and incubated for 2 hours at 50°C. After removal of the filters the assay plates are stained with 10% Lugol solution. Starch degrading variants are detected as white spots on dark blue background and then identified on the storage plates. Positive variants are re-screened twice under the same conditions as the first screen.

Secondary screening

Positive transformants after rescreening are picked from the storage plate and tested in a secondary plate assay. Positive transformants are grown for 22 hours at 37°C in 5 ml LB + chloramphenicol. The *Bacillus* culture of each positive transformant and as a control a clone expressing the corresponding backbone are incubated in citrate buffer, pH 4.5 at 90°C and samples are taken at 0, 10, 20, 30, 40, 60 and 80 minutes. A 3 micro liter sample is spotted on an assay plate. The assay plate is stained with 10% Lugol solution. Improved variants are seen as variants with higher residual activity (detected as halos on the assay plate) than the backbone. The improved variants are determined by nucleotide sequencing.

Stability assay of unpurified variants:

Bacillus cultures expressing the variants to be analysed are grown for 21 hours at 37°C in 10 ml LB+chloramphenicol. 800 micro liter culture is mixed with 200 micro l citrate buffer, pH 4.5. A number of 70 micro l aliquots corresponding to the number of sample time points are made in PCR tubes and incubated at 70°C or 90°C for various time points (typically 5, 10, 15, 20, 25 and 30 minutes) in a PCR machine. The 0 min sample is not incubated at high temperature. Activity in the sample is measured by transferring 20 micro l to 200 micro l of the alpha-amylase PNP-G₇ substrate MPR3 ((Boehringer Mannheim Cat. no. 1660730) as described below under "Assays for Alpha-Amylase Activity". Results are plotted as percentage activity (relative to the 0 time point) versus time, or stated as percentage residual activity after incubation for a certain period of time.

Fermentation and purification of alpha-amylase variants

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

The colonies are transferred to 100 ml PS-1 media supplemented with 10 micro g/ml

chloamphenicol in a 500 ml shaking flask.

Composition of PS-1 medium:

Pearl sugar	100 g/l
Soy Bean Meal	40 g/l
Na ₂ HPO ₄ , 12 H ₂ O	10 g/l
Pluronic™ PE 6100	0.1 g/l
5 CaCO ₃	5 g/l

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. 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 Q-sepharose F.F. and eluted with a linear gradient from 0-0.3M NaCl over 6 column volumes. The fractions that 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.

Stability determination of purified variants

All stability trials of purified variants are made using the same set up. The method is as follows:

20 The enzyme is incubated under the relevant conditions (1-4). Samples are taken at various time points, e.g., after 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 is measured using the Phadebas assay (Pharmacia) under standard conditions pH 7.3, 37°C.

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

Specific activity determination

30 The specific activity is determined using the Phadebas® assay (Pharmacia) as activity/mg enzyme. The manufactures instructions are followed (see also below under "Assay for Alpha-Amylase Activity").

Determination of Isoelectric Point

The pI is determined by isoelectric focusing (ex: Pharmacia, Ampholine, pH 3.5-9.3).

Stability determination

The amylase stability is measured using the method as follows:

The enzyme is incubated under the relevant conditions. Samples are taken at various time points, e.g., after 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 is measured using the Phadebas assay (Pharmacia) under standard conditions pH 7.3, 37°C.

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

Measurement of the calcium- and pH-dependent stability

Normally industrial liquefaction processes 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

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

Two different methods can be used to measure the alterations in stability obtained by the different substitutions in the α -amylase in question:

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

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

Method 2. One assay, which measure the stability in the absence of free calcium and where the pH is maintained at pH 6.0. This assay measures the decrease in calcium sensitivity:

10 micro 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 calcium). Incubation was made in a water bath at 95°C for 30 minutes.

Oxidation stability Determination

Raw filtered culture broths with different vatiants of the invention are diluted to an amylase activity of 100 KNU/ml (defined above) in 50 mM of a Britton-Robinson buffer at pH 9.0 and incubated at 40°C. Subsequently H₂O₂ is added to a concentration of 200 mM, and the pH value is re-adjusted to 9.0. The activity is now measured after 15 seconds and after 5, 15, and 30 minutes. The absorbance of the resulting blue solution, measured spectrophotometrically at

620 nm, is a function of the alpha-amylase activity.

Washing Performance

Washing performance is evaluated by washing soiled test swatches for 15 and 30
5 minutes at 25°C and 40°C, respectively; at a pH in the range from 9-10.5; water hardness in
the range from 6 to 15°dH; Ca:Mg ratio of from 2:1 to 4:1, in different detergent solutions (see
above as described above in the Materials section) dosed from 1 to 5 g/l, such as 3 g/l,
dependent on the detergent with the alpha-amylase variant in question.

The recombinant alpha-amylase variant is added to the detergent solutions at
10 concentrations of for instance 0.01-5 mg/l. The test swatches are soiled with orange rice
starch (CS-28 swatches available from CFT, Center for Test Material, Holland).

After washing, the swatches are evaluated by measuring the remission at 460 nm using
an Elrepho Remission Spectrophotometer. The results are expressed as DeltaR = remission
(°R of the swatch washed with the alpha-amylase minus the remission of a swatch washed at
15 the same conditions without the alpha-amylase.

Assays for Alpha-Amylase Activity

1. Phadebas assay

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

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
25 CaCl₂, pH adjusted to the value of interest with NaOH). The test is performed in a water bath at
the temperature of interest. The alpha-amylase to be tested is diluted in x ml of 50 mM Britton-
Robinson buffer. 1 ml of this alpha-amylase solution is added to the 5 ml 50 mM Britton-
Robinson buffer. The starch is hydrolyzed by the alpha-amylase giving soluble blue fragments.
The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a
30 function of the alpha-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
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
35 (temp., pH, reaction time, buffer conditions) 1 mg of a given alpha-amylase will hydrolyze a

certain amount of substrate and a blue colour will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure alpha-amylase protein) of the alpha-amylase in question under the given set of conditions.

5

2. Alternative method

Alpha-amylase activity is determined by a method employing the PNP-G₇ substrate. PNP-G₇ which is a abbreviation for p-nitrophenyl-alpha,D-maltoheptaoside is a blocked oligosaccharide which can be cleaved by an endo-amylase. Following the cleavage, the alpha-Glucosidase included in the kit digest the substrate to liberate a free PNP molecule which has a yellow colour and thus can be measured by visible spectrophotometry at $\lambda=405\text{nm}$ (400-420 nm). Kits containing PNP-G₇ substrate and alpha-Glucosidase is manufactured by Boehringer-Mannheim (cat. No.1054635).

To prepare the reagent solution 10 ml of substrate/buffer solution is added to 50 ml enzyme/buffer solution as recommended by the manufacturer. The assay is performed by transferring 20 micro l sample to a 96 well microtitre plate and incubating at 25°C. 200 micro l reagent solution pre-equilibrated to 25°C is added. The solution is mixed and pre-incubated 1 minute and absorption is measured every 30 sec. over 4 minutes at OD 405 nm in an ELISA reader.

The slope of the time dependent absorption-curve is directly proportional to the activity of the alpha-amylase in question under the given set of conditions.

Determination of LAS sensitivity

The variant is incubated with different concentrations of LAS (linear alkyl benzene sulphonate; Nansa 1169/P) for 10 minutes at 40°C.

The residual activity is determined using the Phadebas® assay method or the alternative method employing the PNP-G₇ substrate.

LAS is diluted in 0.1 M phosphate buffer pH 7.5.

The following concentrations are used:

500 ppm, 250 ppm, 100 ppm, 50ppm, 25ppm, and 10 ppm on no LAS.

The variant is diluted in the different LAS buffers to concentration of 0.01-5 mg/l in a total volume of 10 ml and incubated for 10 minutes in a temperature controlled water bath. The incubation is stopped by transferring a small aliquat into cold assay buffer. It is important that during activity measurement the LAS concentration is below 1 ppm, in order not to affect the activity measurement.

Then the residual activity is determined in duplicate using the above mentioned Phadebas® assay or alternative method.

The activity is measured after subtraction of the blank.

The activity with no LAS is 100%.

5

EXAMPLES

Example 1.

Construction of variants of the invention and determination of altered properties

10 The below listed variants are constructed as described in EXAMPLE 1 of WO 00/29560 (from Novozymes A/S) in the parent *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 8.

G5A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;

T6A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
G36A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
I37A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V;
T38A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
5 A39R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
I42A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V;
A45R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K47A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
D63A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
10 E66A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Q69A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K70A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
G71A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
T72A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
15 R74A,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
T75A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
K76A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
T79A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
E82A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
20 L83A,R,N,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
A86R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
I87A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V;
S89A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
R93A,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
25 T112A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
E113A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
A117R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
V120A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
A137R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
30 K213A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
G216A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
A220R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L223A,R,N,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
L225A,R,N,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
35 D226A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G227A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;

R229A,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D243A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
V245A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
F279A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V;
5 S282A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
T311A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
V321A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
V324A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
L352A,R,N,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
10 T353A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
R354A,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G357A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
V361A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
F362A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V;
15 G364A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
G368A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
A390R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
A395R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G397A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
20 Q399A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
H400A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
D401A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
A425R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D451A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
25 I452A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V;
T453A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
G466A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
G468A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
F470A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V;
30 H471A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
S478A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V
L7A,R,N,D,C,Q,E,G,H,K,M,P,S,Y,V;
M8C;
Q9A,R,N,D,C,G,H,M,P,S,T,W,Y,V;
35 F11A,N,D,C,Q,G,H,I,L,M,P,S,T,W,Y,V;
E12A,R,N,D,C,G,H,I,L,K,M,F,P,S,T,W,Y,V;

- G19A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
H21A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
W22A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,Y,V;
L25A,R,N,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
5 L32A,R,N,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
V40A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
W41A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,Y,V;
Y46A,R,D,C,G,K,M,P,W;
G48R,N,D,C,Q,E,H,K,M,F,P,W,Y;
10 G55A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
G57R,N,D,C,Q,E,H,K,M,P,W;
A58R,N,D,C,Q,E,G,H,K,M,S,T,W,Y;
D60A,R,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Y77A,R,D,C,G,K,M,P,W;
15 I95A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V;
V97A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
Y98A,R,D,C,G,K,M,P,W;
G99R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
D100A,R,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
20 V101A,N,C,Q,G,I,L,M,P,S,T,W,Y;
V102N,D,C,Q,E,H,I,L,M,F,P,W,Y;
I103A,N,D,C,Q,E,G,M,P,S,W,Y;
H105A,N,C,Q,G,I,L,M,P,S,T,Y,V;
G107R,N,D,Q,E,H,K,M,F,P,W,Y;
25 V115R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y;
V118R,N,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y;
I135A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V;
T139A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
F141A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V;
30 F143A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,Y,V;
S151A,R,N,D,C,Q,E,G,H,K,M,P,T,Y,V;
H159A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,V;
F160A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V;
D161A,R,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
35 G162A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
T163A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;

- D166A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Y175A,R,D,C,G,K,M,P,W;
F177A,N,D,C,Q,E,H,I,L,K,M,P,S,T,W,Y,V;
D183A,R,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
5 V186A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
S187A,R,C,Q,E,G,H,I,L,K,M,F,P,W,Y,V;
N192A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
A199R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D200A,R,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
10 D202A,R,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Y203A,R,D,C,G,K,M,P;
V208A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y;
I212A,N,C,Q,G,H,M,P,S,T,V;
W215A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,Y,V;
15 Y219A,R,D,C,G,K,M,P,W;
F228A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V;
L230A,R,N,D,C,Q,E,G,M,P,S,T,W,Y,V;
V233R,N,C,Q,E,G,H,I,K,M,P,S,T,W,Y;
I236A,C,Q,G,H,M,P,S,T,V;
20 F238A,R,N,D,C,Q,E,G,H,I,K,M,P,S,T,W,Y,V;
F240A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V;
L241A,N,C,Q,G,H,P,S,T,V;
W244A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,Y,V;
V248A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
25 M256C;
T258A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
V259A,R,N,D,C,Q,E,G,H,K,M,P,S,T,W,Y;
A260R,N,D,C,Q,E,H,I,L,K,M,F,P,T,W,Y,V;
Y262A,R,D,C,G,K,M,P,W;
30 L270A,N,C,Q,G,I,M,F,P,S,T,W,Y,V;
Y273A,R,D,C,G,K,M,P;
L274A,R,N,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
T277A,R,N,D,C,Q,E,G,H,K,M,P,S,W,Y,V;
H281A,R,N,D,C,Q,E,G,K,M,P,S,T,W,Y,V;
35 V283A,R,N,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y;
F284A,R,N,D,C,Q,E,G,I,L,K,M,P,S,T,Y,V;

- D285A,R,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
V286A,R,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y;
P287R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;
L288A,R,N,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
5 H289A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
F292A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V;
A295R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
S296A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
M304C;
10 L307A,R,N,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
V312A,N,C,Q,G,H,I,L,M,F,P,S,T,W,Y;
V313A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
S320R,N,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
T322R,N,D,C,Q,E,G,H,L,K,M,F,P,S,W,Y,V;
15 F323A,R,N,D,C,Q,E,G,I,L,K,M,P,S,T,W,Y,V;
D325A,R,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
N326A,R,C,Q,E,G,M,P,S,T,W;
H327A,R,C,G,I,L,K,M,P,S,T,W,Y,V;
T329A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,W,Y,V;
20 P331N,D,C,Q,E,G,H,I,L,M,F,S,T,W,Y,V;
V339A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
F343A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,Y,V;
K344A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
L346A,R,N,D,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
25 A347R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
A349R,N,D,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F350A,R,N,C,Q,G,H,I,L,K,M,P,S,T,Y,V;
P359R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;
Q360N,D,G,H,I,L,M,F,P,S,T,W,Y,V;
30 T369A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
I377A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V;
L380A,R,N,D,C,Q,E,G,H,K,P,S,W,Y,V;
I387A,R,N,D,C,Q,E,G,H,L,K,M,P,S,T,W,Y,V;
V409N,C,Q,E,G,H,M,P,S,T,W,Y;
35 G410A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
W411A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,Y,V;

- T412R,N,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
G423A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
L424A,R,N,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
A426R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
5 L427A,R,N,D,C,Q,E,G,H,K,M,P,S,T,Y,V;
I428A,N,D,Q,E,G,H,M,F,P,S,W,Y,V;
T429A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
D430A,R,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
M438C;
10 V440R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
G441A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
W449A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,Y,V;
I462A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V;
V472A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
15 V477A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
I479A,R,N,D,Q,E,G,H,L,K,M,F,P,S,W,Y,V;
Y480A,R,D,C,G,K,M,P,W;
V481A,R,N,D,C,Q,E,G,H,K,M,P,S,T,Y.
A1R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;
20 N2R,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;
L3A,R,N,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y;
N4A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W13R,N,D,C,Q,E,G,H,K,M,P,S,T;
Y14A,R,D,C,G,K,M,P,W;
25 P16R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;
N17A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D18A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Q20A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
R23A,N,D,C,Q,E,G,H,I,L,M,F,P,S,W,Y,V;
30 R24A,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Q26A,D,C,E,G,H,I,L,M,F,P,S,T,W,V;
E34A,R,N,C,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
H35A,R,N,D,C,Q,E,G,K,M,F,P,S,T,W,Y,V;
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35 S50A,R,N,C,Q,E,G,H,K,M,F,P,W;
Q51A,N,D,C,E,G,H,I,L,M,F,P,S,T,W,Y,V;

- A52R,D,C,Q,E,G,H,K,P;
D53A,C,G,H,K,M,P;
L61A,R,N,D,C,Q,E,G,H,I,K,M,P,S,T,Y;
Y62A,R,D,C,G,K,M,P;
5 F67A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,Y,V;
H68A,R,D,C,E,G,I,L,K,M,F,P,S,T,W,Y,V;
V73A,R,N,D,C,Q,E,G,H,K,M,P,S,T,W,Y;
Q84A,R,N,D,C,G,H,I,L,K,M,F,P,S,T,W,Y,V;
S85A,R,N,C,E,G,H,I,L,K,M,F,P,T,W,Y,V;
10 K88A,R,N,D,C,E,G,H,I,L,M,F,P,S,T,W,Y,V;
H91A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
S92A,R,N,D,C,Q,E,G,H,I,L,M,F,P,T,W,Y,V;
N96A,R,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K106A,N,D,C,Q,E,G,H,I,L,M,P,S,T,Y,V;
15 G108R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
D114A,N,C,Q,E,G,H,K,F,P,S,T,W,Y;
T116A,R,D,C,Q,E,G,H,I,L,M,F,P,S,W,Y,V;
E119A,R,N,D,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D121A,R,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
20 P122R,N,Q,G,H,I,L,M,F,S,T,W,Y,V;
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D124N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
R125N,Q,E,G,I,K,M,F,S,T,W,Y;
N126Q,G,H,I,L,M,F,P,S,T,W,Y,V;
25 R127A,N,D,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
V128A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,W,Y;
I129A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,W,Y,V;
S130A,R,N,D,Q,E,G,H,I,L,K,M,F,P,W,Y,V;
G131A,R,N,D,C,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
30 E132R,N,D,C,Q,G,H,I,L,K,M,F,S,W,Y;
H133R,N,D,C,M,T,W,V;
L134A,N,D,C,E,G,H,I,K,M,F,P,S,T,W,Y,V;
K136A,R,N,D,C,E,G,H,I,L,M,F,P,S,T,W,Y,V;
W138R,N,D,Q,E,G,I,K,M,P,S,T,V;
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- S148A,R,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
T149A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,W,Y,V;
Y150A,D,C,G,M,P,W;
D152A,R,N,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
5 F153A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V;
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H156A,C,Q,E,G,I,L,M,F,P,S,T,W,V;
W157R,I,L,M,F,P,S,T,Y,V;
10 Y158R,M,P,W;
D164R,I,L,M,F,P,S,T,W,Y,V;
W165A,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,Y,V;
E167A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
S168A,R,N,D,C,Q,E,G,H,I,L,K,M,F,T,W,V;
15 R169A,N,D,C,Q,E,G,H,M,P,S,W,Y,V;
K170A,R,N,D,C,E,G,H,I,L,M,F,P,S,T,W,Y,V;
L171A,R,N,D,C,Q,E,G,H,K,M,P,S,T,W,Y,V;
N172A,D,C,Q,E,G,I,L,M,F,P,T,W,Y,V;
R173A,N,D,C,Q,E,G,H,M,P,S,W,Y,V;
20 K176A,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
G179D,C,Q,E,H,I,L,K,M,F,P,W,Y,V;
K180A,G,I,L,M,F,P,W,Y,V;
A181G;
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25 W184I,L,M,F,P,W,Y,V;
E185R,I,L,M,F,P,S,T,W,Y,V;
N188A,R,Q,G,H,L,M,F,W,V;
E189A,R,N,G,H,I,L,M,F,P,S,T,W,Y,V;
G191A,R,N,Q,H,I,L,M,F,P,S,T,W,Y,V;
30 Y193A,R,G,M,P,W;
L196A,N,Q,G,H,I,M,P,S,T,W,V;
Y198A,R,G,M,P,W;
D204R,L,M,F,P,T,W,Y,V;
H205A,G,I,L,M,F,P,W,Y,V;
35 P206R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;
A209R,P,S,W,Y;

- A210R,N,D,C,Q,E,G,H,I,L,K,M,F,P,W,Y,V;
R214A,D,C,Q,E,G,I,L,M,F,P,S,T,Y,V;
T217A,R,N,D,C,Q,G,H,I,L,M,F,P,S,W,Y;
W218A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,Y,V;
5 N221A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
E222A,R,N,D,C,G,H,I,L,K,M,F,P,S,W,Y,V;
K234A,D,C,G,H,I,M,F,P,S,T,W,Y,V;
H235A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
K237A,G,H,I,L,M,F,W,Y,V;
10 S239G,H,I,L,M,F,P,T,Y,V;
R242G,I,L,M,F,S,T,W,Y,V;
N246A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
H247R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,V;
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15 E250A,R,N,D,C,H,I,L,K,M,P,T,W,Y,V;
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T252A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
G253R,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y;
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20 E255A,R,D,C,G,H,I,L,K,M,F,S,T,W,Y,V;
F257A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V;
E261A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W263A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,Y,V;
N265C,Q,E,H,I,L,M,F,P,W;
25 D266A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L267A,R,N,D,C,Q,E,G,H,K,P,S,T,W,Y,V;
G268A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
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30 N272A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
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Q291A,R,N,G,H,I,L,M,F,P,S,T,W,Y,V;

- H293A,R,N,G,I,L,M,P,S,T,W,V;
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5 G299A,H,M,P,S,T;
G300A,C,H,I,L,M,F,P,T,W,Y,V;
G301N,Q,H,I,L,M,F,P,S,T,W,Y,V;
Y302R,M,P,W;
D303R,I,L,M,F,P,S,T,W,Y,V;
10 R305G,I,L,M,F,P,S,T,W,Y,V;
K306Q,G,H,I,L,M,F,P,S,T,W,Y,V;
L308A,R,N,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
N309Q,G,H,I,L,M,F,P,S,T,W,Y,V;
G310A,R,N,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
15 S314A,D,C,E,G,I,L,M,F,P,W,Y,V;
K315A,N,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
H316A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,V;
P317R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;
L318A,R,N,D,C,Q,E,G,H,I,K,P,S,W,Y,V;
20 K319A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
D328A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G332A,R,N,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
Q333A,N,D,C,G,I,M,F,P,S,T,Y,V;
S334R,N,C,Q,E,G,H,K,M,F,P,W,Y;
25 L335R,D,C,Q,E,H,I,K,M,F,P,W,Y,V;
E336A,N,D,C,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
S337A,R,N,C,Q,E,G,H,I,L,M,F,P,T,W,Y,V;
T338A,R,N,C,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
Q340I,L,M,F,P,S,T,W,Y,V;
30 T341A,R,D,C,G,H,I,L,K,M,F,W,Y,V;
W342R,I,L,M,F,P,S,T,Y,V;
P345R,N,D,C,Q,E,G,H,I,L,K,M,F,T,W,Y,V;
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35 Y363A,R,D,C,G,K,M,P,W;
K370A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;

- G371A,N,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
S373A,R,N,D,C,Q,E,G,H,I,L,K,M,F,T,W,Y,V;
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R375A,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,V;
5 E376A,R,N,D,C,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
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10 Q393A,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
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Y396A,R,D,C,G,K,M,P,W;
A398R,N,D,C,Q,E,G,H,I,L,M,F,W,Y,V;
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15 F403A,R,N,Q,G,H,I,L,M,P,S,T,W,Y,V;
D404R,I,L,M,F,P,S,T,W,Y,V;
H405R,G,I,L,M,F,P,W,Y,V;
H406A,R,G,I,M,F,P,Y,V;
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20 I408A,R,N,D,C,Q,E,G,H,K,M,P,S,T,W,Y,V;
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25 S417A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,W,Y,V;
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A420N,D,C,E,G,H,I,L,K,M,F,S,T,W,Y,V;
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30 S422A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
G431A,R,N,Q,H,I,L,M,F,P,S,T,W,Y,V;
P432G,H,I,L,M,F,S,T,W,Y,V;
G433A,R,N,Q,H,I,L,M,F,P,S,T,W,Y,V;
G434A,R,N,Q,H,I,L,M,F,P,S,T,W,Y,V;
35 A435Q,G,H,I,L,M,F,P,T,W,Y,V;
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R437A,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

Y439A,R,D,C,G,K,M,P,W;

R442A,N,D,C,E,G,H,I,L,M,F,P,S,T,W,Y,V;

Q443A,R,N,D,C,E,G,H,I,L,M,F,P,S,T,W,Y,V;

5 N444A,C,G,H,I,L,M,F,P,S,T,W,Y,V;

A445R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

G446A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

E447A,N,D,C,G,H,I,L,M,F,P,S,T,W,Y,V;

T448A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;

10 H450A,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,V;

G454A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;

N455A,R,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

R456A,D,C,E,G,H,I,L,M,F,P,S,T,W,Y,V;

S457A,R,N,D,C,Q,E,G,H,I,L,K,M,F,W,Y,V;

15 E458A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

P459R,N,D,C,Q,E,G,H,I,L,K,M,F,S,W,Y,V;

V460A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;

V461A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,W,Y;

N463A,R,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;

20 S464A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;

E465A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

W467A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,Y,V;

E469A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

N473Q,G,H,I,L,M,F,P,S,T,W,Y,V;

25 G474A,R,H,I,L,M,F,P,W,Y,V;

G475A,N,Q,H,I,L,M,F,P,S,T,W,Y,V;

S476G,H,I,L,M,F,P,T,W,Y,V;

Q482A,N,D,C,G,H,I,L,M,F,S,T,W,Y,V;

R483A,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V.

30 The variants are tested for altered substrate specificity, substrate binding, substrate cleavage pattern, thermal stability, pH activity profile, pH stability profile, stability towards oxidation, Ca^{2+} dependency, reduced LAS sensitivity, reduced and increased pl and improved wash performance, and specific activity as described in the "Materials & Methods" section above.

35

Example 2.

Construction of variants of the invention and determination of altered properties

The below listed variants are constructed as described in EXAMPLE 1 of WO 00/37626 (from Novozymes A/S) in the parent *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 8. The alterations of the variants are, as specified in the list below, insertion of an amino acid downstream of the amino acid which occupies the position, or deletion of the amino acid which occupies the position.

A1 insertion;

L3 insertion;

N4 insertion;

10 N17 insertion;

D18 insertion;

Q20 insertion;

R23 insertion;

R24 insertion;

15 D28 insertion;

Y56 insertion;

L61 insertion or deletion;

Y62 insertion;

F67 insertion or deletion;

20 H68 insertion;

K80 insertion or deletion;

G81 insertion or deletion;

Q84 insertion;

S85 insertion;

25 H91 insertion or deletion;

S92 insertion or deletion;

K106 insertion or deletion;

D110 insertion or deletion;

D114 deletion;

30 E119 insertion or deletion;

D121 insertion;

P122 insertion;

A123 insertion;

D124 insertion;

35 R125 insertion;

N126 insertion;

R127 insertion;
I129 insertion;
G131 insertion;
L134 insertion;
5 K136 insertion;
N172 insertion;
E185 insertion;
L196 insertion or deletion;
P206 insertion or deletion;
10 T217 insertion;
W218 insertion;
D231 insertion or deletion;
A232 insertion or deletion;
H235 insertion or deletion;
15 N246 insertion;
H247 insertion;
R249 insertion;
K251 insertion;
F257 insertion or deletion;
20 N278 insertion;
G310 insertion or deletion;
H316 insertion;
P317 insertion;
D328 insertion or deletion;
25 G332 insertion or deletion;
E355 insertion or deletion;
Y358 insertion;
Y363 insertion;
Y367 insertion;
30 K370 insertion;
S373 insertion;
R375 insertion;
E376 insertion;
K381 insertion;
35 H382 insertion;
R391 insertion or deletion;

Y396 insertion;
R413 insertion or deletion;
E414 insertion or deletion;
G415 insertion or deletion;
5 D416 insertion;
S417 insertion;
S418 insertion;
V419 insertion;
A420 insertion;
10 N421 insertion;
S422 insertion or deletion;
Y439 insertion;
A445 insertion or deletion;
G446 insertion or deletion;
15 T448 insertion or deletion;
H450 insertion;
G454 insertion or deletion;
N455 insertion;
E458 insertion;
20 P459 insertion;
V460 insertion;
V461 insertion;
N463 insertion;
S464 insertion;
25 E465 insertion;
W467 insertion;
L7 insertion or deletion;
M8 insertion;
Y10 insertion;
30 F11 insertion;
E12 insertion or deletion;
M15 insertion;
G19 insertion;
H21 insertion;
35 W22 insertion;
L25 insertion;

- V40 insertion or deletion;
W41 insertion;
P43 insertion or deletion;
P44 insertion or deletion;
5 Y46 insertion;
G55 insertion;
Y59 insertion;
Y77 insertion;
G78 insertion or deletion;
10 L90 insertion or deletion;
I95 insertion;
V97 insertion;
Y98 insertion;
G99 insertion;
15 D100 insertion;
V101 insertion;
V102 insertion;
H105 insertion or deletion;
A109 insertion or deletion;
20 V115 insertion or deletion;
V118 insertion or deletion;
I135 insertion;
T139 insertion or deletion;
F141 insertion or deletion;
25 Y195 insertion;
V208 insertion or deletion;
W215 insertion;
Y219 insertion;
I236 insertion or deletion;
30 F238 insertion or deletion;
F240 insertion or deletion;
W244 insertion;
V248 insertion;
M256 insertion;
35 T258 insertion or deletion;
V259 insertion or deletion;

- V312 insertion or deletion;
V313 insertion or deletion;
S320 insertion;
T322 insertion or deletion;
5 F323 insertion or deletion;
D325 insertion or deletion;
N326 insertion;
H327 insertion or deletion;
Q330 insertion or deletion;
10 P331 insertion or deletion;
Y348 insertion;
A349 insertion or deletion;
F350 insertion or deletion;
P359 insertion or deletion;
15 Q360 insertion;
D365 insertion or deletion;
M366 insertion;
T369 insertion;
I377 insertion;
20 I384 insertion or deletion;
L388 insertion or deletion;
G423 insertion or deletion;
L424 insertion or deletion;
M438 insertion;
25 G441 insertion or deletion;
W449 insertion;
I462 insertion;
I479 insertion or deletion;
Y480 insertion;
30 V481 insertion or deletion.

The variants are tested for altered substrate specificity, substrate binding, substrate cleavage pattern, thermal stability, pH activity profile, pH stability profile, stability towards oxidation, Ca^{2+} dependency, reduced and increased pI and improved wash performance, specific activity as described in the "Materials & Methods" section above.

CLAIMS

1. A variant of a parent Termamyl-like alpha-amylase, comprising an alteration at one or more positions selected from the group of:

5 5, 6, 36, 37, 38, 39, 42, 45, 47, 63, 66, 69, 70, 71, 72, 74, 75, 76, 79, 82, 83, 86, 87, 89, 93, 112, 113, 117, 120, 137, 213, 216, 220, 223, 225, 226, 227, 229, 243, 245, 279, 282, 311, 321, 324, 352, 353, 354, 357, 361, 362, 364, 368, 390, 395, 397, 399, 400, 401, 425, 451, 452, 453, 466, 468, 470, 471, 478,

wherein

10 (a) the alteration(s) are independently

(i) an insertion of an amino acid downstream of the amino acid which occupies the position,

(ii) a deletion of the amino acid which occupies the position, or

(iii) a substitution of the amino acid which occupies the position with a different amino acid,

15 (b) the variant has alpha-amylase activity and (c) each position corresponds to a position of the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8 (*Bacillus licheniformis* alpha-amylase).

2. A variant of a parent Termamyl-like alpha-amylase, comprising one or more of the following substitutions:

20 X1A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;

X2R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;

X3A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y;

X4A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

25 X13R,N,D,C,Q,E,G,H,K,M,P,S,T,W;

X14A,R,D,C,G,K,M,P,W;

X16R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;

X17A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

X18A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

30 X20A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

X23A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,W,Y,V;

X24A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

X26A,D,C,E,G,H,I,L,M,F,P,S,T,W,V;

X34A,R,N,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;

35 X35A,R,N,D,C,Q,E,G,H,K,M,F,P,S,T,W,Y,V;

X49A,C,G,H,P,T;

- X50A,R,N,C,Q,E,G,H,K,M,F,P,S,W;
X51A,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X52A,R,D,C,Q,E,G,H,K,P;
X53A,D,C,G,H,K,M,P;
5 X61A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,Y;
X62A,R,D,C,G,K,M,P,Y;
X67A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,Y,V;
X68A,R,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X73A,R,N,D,C,Q,E,G,H,K,M,P,S,T,W,Y,V;
10 X84A,R,N,D,C,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X85A,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X88A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X91A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X92A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
15 X96A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X106A,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,Y,V;
X108R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X114A,N,C,Q,E,G,H,K,F,P,S,T,W,Y;
X116A,R,D,C,Q,E,G,H,I,L,M,F,P,S,W,Y,V;
20 X119A,R,N,D,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X121A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X122R,N,Q,G,H,I,L,M,F,S,T,W,Y,V;
X123N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X124N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
25 X125R,N,Q,E,G,I,K,M,F,S,T,W,Y;
X126N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X127A,R,N,D,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X128A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
X129A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
30 X130A,R,N,D,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
X131A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X132R,N,D,C,Q,E,G,H,I,L,K,M,F,S,W,Y;
X133R,N,D,C,M,T,W,V;
X134A,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
35 X136A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X138R,N,D,Q,E,G,I,K,M,P,S,T,W,V;

- X145A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,Y,V;
X147A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V;
X148A,R,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
X149A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V;
5 X150A,D,C,G,M,P,W,Y;
X152A,R,N,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
X153A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X154A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X155A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
10 X156A,C,Q,E,G,I,L,M,F,P,S,T,W,V;
X157R,I,L,M,F,P,S,T,W,Y,V;
X158R,M,P,W,Y;
X164R,I,L,M,F,P,S,T,W,Y,V;
X165A,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
15 X167A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X168A,R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,V;
X169A,R,N,D,C,Q,E,G,H,M,P,S,W,Y,V;
X170A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X171A,R,N,D,C,Q,E,G,H,K,M,P,S,T,W,Y,V;
20 X172A,N,D,C,Q,E,G,I,L,M,F,P,T,W,Y,V;
X173A,N,D,C,Q,E,G,H,M,P,S,W,Y,V;
X176A,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X179D,C,Q,E,H,I,L,K,M,F,P,W,Y,V;
X180A,G,I,L,M,F,P,W,Y,V;
25 X181G;
X182A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X184I,L,M,F,P,W,Y,V;
X185R,I,L,M,F,P,S,T,W,Y,V;
X188A,R,N,Q,G,H,L,M,F,W,V;
30 X189A,R,N,G,H,I,L,M,F,P,S,T,W,Y,V;
X190N;
X191A,R,N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X193A,R,G,M,P,W,Y;
X196A,N,Q,G,H,I,L,M,P,S,T,W,V;
35 X198A,R,G,M,P,W;
X204R,L,M,F,P,T,W,Y,V;

- X205A,G,H,I,L,M,F,P,W,Y,V;
X206R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X209R,P,S,W,Y;
X210A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,W,Y,V;
5 X211E;
X214A,D,C,Q,E,G,I,L,M,F,P,S,T,Y,V;
X217A,R,N,D,C,Q,G,H,I,L,M,F,P,S,T,W,Y;
X218A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X221A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
10 X222A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,W,Y,V;
X234A,D,C,G,H,I,K,M,F,P,S,T,W,Y,V;
X235A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X237A,G,H,I,L,M,F,W,Y,V;
X239G,H,I,L,M,F,P,S,T,Y,V;
15 X242G,I,L,M,F,S,T,W,Y,V;
X246A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X247R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,V;
X249A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X250A,R,N,D,C,E,H,I,L,K,M,P,T,W,Y,V;
20 X251R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X252A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
X253R,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y;
X254A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X255A,R,D,C,G,H,I,L,K,M,F,S,T,W,Y,V;
25 X257A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X261A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X263A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
X265C,Q,E,H,I,L,M,F,P,W;
X266A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
30 X267A,R,N,D,C,Q,E,G,H,K,P,S,T,W,Y,V;
X268A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X269A,N,C,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X271A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X272A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
35 X275A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
X276A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;

- X278A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X280A,R,D,C,E,G,H,I,L,K,M,F,P,W,Y,V;
X290W,Y;
X291A,R,N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
5 X293A,R,N,G,I,L,M,P,S,T,W,V;
X294R,N,Q,G,H,I,L,M,F,P,S,T,W,Y;
X297A,G,H,I,L,M,F,P,W,Y,V;
X298G,H,I,L,M,F,P,S,T,W,Y,V;
X299A,G,H,M,P,S,T;
10 X300A,C,G,H,I,L,M,F,P,T,W,Y,V;
X301N,Q,H,I,L,M,F,P,S,T,W,Y,V;
X302R,M,P,W,Y;
X303R,I,L,M,F,P,S,T,W,Y,V;
X305G,I,L,M,F,P,S,T,W,Y,V;
15 X306Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X308A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X309N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X310A,R,N,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
X314A,D,C,E,G,I,L,M,F,P,W,Y,V;
20 X315A,N,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X316A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,V;
X317R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X318A,R,N,D,C,Q,E,G,H,I,K,P,S,W,Y,V;
X319A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
25 X328A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X332A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X333A,N,D,C,G,I,M,F,P,S,T,Y,V;
X334R,N,C,Q,E,G,H,K,M,F,P,S,W,Y;
X335R,D,C,Q,E,H,I,L,K,M,F,P,W,Y,V;
30 X336A,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X337A,R,N,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X338A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X340I,L,M,F,P,S,T,W,Y,V;
X341A,R,D,C,G,H,I,L,K,M,F,W,Y,V;
35 X342R,I,L,M,F,P,S,T,W,Y,V;
X345R,N,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;

- X355A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X358A,R,D,C,G,K,M,P,W,Y;
X363A,R,D,C,G,K,M,P,W,Y;
X370A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
5 X371A,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X373A,R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;
X374A,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;
X375A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,V;
X376A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
10 X378R,N,D,C,Q,E,G,H,I,L,K,M,F,S,T,W,Y,V;
X379A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
X381A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X389A,D,C,G,H,M,F,P,S,T,W,Y,V;
X393A,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
15 X394A,R,D,C,G,K,M,P,W,Y;
X396A,R,D,C,G,K,M,P,W,Y;
X398A,R,N,D,C,Q,E,G,H,I,L,M,F,W,Y,V;
X402R,C,G,K,M,P,W,Y;
X403A,R,N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
20 X404R,I,L,M,F,P,S,T,W,Y,V;
X405R,G,H,I,L,M,F,P,W,Y,V;
X406A,R,G,H,I,M,F,P,Y,V;
X407A,R,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X408A,R,N,D,C,Q,E,G,H,I,K,M,P,S,T,W,Y,V;
25 X413A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X414A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X415A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X416A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X417A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
30 X418A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X419A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
X420A,N,D,C,E,G,H,I,L,K,M,F,S,T,W,Y,V;
X421A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
X422A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
35 X431A,R,N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X432G,H,I,L,M,F,P,S,T,W,Y,V;

- X433A,R,N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 X434A,R,N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 X435Q,G,H,I,L,M,F,P,T,W,Y,V;
 X436A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 5 X437A,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X439A,R,D,C,G,K,M,P,W,Y;
 X442A,R,N,D,C,E,G,H,I,L,M,F,P,S,T,W,Y,V;
 X443A,R,N,D,C,E,G,H,I,L,M,F,P,S,T,W,Y,V;
 X444A,C,G,H,I,L,M,F,P,S,T,W,Y,V;
 10 X445A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X446A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X447A,N,D,C,G,H,I,L,M,F,P,S,T,W,Y,V;
 X448A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X450A,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,V;
 15 X454A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X455A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X456A,D,C,E,G,H,I,L,M,F,P,S,T,W,Y,V;
 X457A,R,N,D,C,Q,E,G,H,I,L,K,M,F,W,Y,V;
 X458A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 20 X459R,N,D,C,Q,E,G,H,I,L,K,M,F,S,W,Y,V;
 X460A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X461A,R,N,D,C,Q,E,G,H,I,L,M,F,P,S,W,Y,V;
 X463A,R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
 X464A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 25 X465A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X467A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X469A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X473N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 X474A,R,G,H,I,L,M,F,P,W,Y,V;
 30 X475A,N,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 X476G,H,I,L,M,F,P,S,T,W,Y,V;
 X482A,N,D,C,G,H,I,L,M,F,S,T,W,Y,V;
 X483A,N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V

wherein

- 35 (a) the variant has alpha-amylase activity and (b) each position corresponds to a position of the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid

sequence shown in SEQ ID NO: 8 (*Bacillus licheniformis* alpha-amylase).

3. A variant of a parent Termamyl-like alpha-amylase, comprising one or more of the following substitutions:

- 5 X7A,R,N,D,C,Q,E,G,H,K,M,P,S,Y,V;
X8C,M
X9A,R,N,D,C,Q,G,H,M,P,S,T,W,Y,V;
X11A,N,D,C,Q,G,H,I,L,M,P,S,T,W,Y,V;
X12A,R,N,D,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
10 X19A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X21A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X22A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X25A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X32A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
15 X40A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X41A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X46A,R,D,C,G,K,M,P,W,Y;
X48R,N,D,C,Q,E,G,H,K,M,F,P,W,Y;
X55A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
20 X57R,N,D,C,Q,E,G,H,K,M,P,W;
X58A,R,N,D,C,Q,E,G,H,K,M,S,T,W,Y;
X60A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X77A,R,D,C,G,K,M,P,W,Y;
X95A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
25 X97A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X98A,R,D,C,G,K,M,P,W,Y;
X99R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X100A,R,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
X101A,N,C,Q,G,I,L,M,P,S,T,W,Y,V;
30 X102N,D,C,Q,E,H,I,L,M,F,P,W,Y,V;
X103A,N,D,C,Q,E,G,M,P,S,W,Y;
X105A,N,C,Q,G,H,I,L,M,P,S,T,Y,V;
X107R,N,D,Q,E,H,K,M,F,P,W,Y;
X115R,N,D,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
35 X118R,N,C,Q,E,H,I,L,K,M,F,P,S,T,W,Y,V;
X135A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

- X139A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X141A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X143A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,Y,V;
X151A,R,N,D,C,Q,E,G,H,K,M,P,S,T,Y,V;
5 X159A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,V;
X160A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X161A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X162A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X163A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
10 X166A,R,N,D,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X175A,R,D,C,G,K,M,P,W,Y;
X177A,N,D,C,Q,E,H,I,L,K,M,P,S,T,W,Y,V;
X183A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X186A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
15 X187A,R,C,Q,E,G,H,I,L,K,M,F,P,W,Y,V;
X192A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X199R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X200A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X202A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
20 X203A,R,D,C,G,K,M,P;
X208A,R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V;
X212A,N,C,Q,G,H,M,P,S,T,V;
X215A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X219A,R,D,C,G,K,M,P,W,Y;
25 X228A,R,N,D,C,Q,E,G,H,I,L,K,M,P,S,T,W,Y,V;
X230A,R,N,D,C,Q,E,G,L,M,P,S,T,W,Y,V;
X233R,N,C,Q,E,G,H,I,K,M,P,S,T,W,Y;
X236A,C,Q,G,H,I,M,P,S,T,V;
X238A,R,N,D,C,Q,E,G,H,I,K,M,P,S,T,W,Y,V;
30 X240A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X241A,N,C,Q,G,H,P,S,T,V;
X244A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X248A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X256C,M;
35 X258A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X259A,R,N,D,C,Q,E,G,H,K,M,P,S,T,W,Y,V;

- X260R,N,D,C,Q,E,H,I,L,K,M,F,P,T,W,Y,V;
X262A,R,D,C,G,K,M,P,W,Y;
X270A,N,C,Q,G,I,L,M,F,P,S,T,W,Y,V;
X273A,R,D,C,G,K,M,P,Y;
5 X274A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X277A,R,N,D,C,Q,E,G,H,K,M,P,S,W,Y,V;
X281A,R,N,D,C,Q,E,G,K,M,P,S,T,W,Y,V;
X283A,R,N,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
X284A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,Y,V;
10 X285A,R,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;
X286A,R,D,C,Q,E,G,H,I,K,M,F,P,S,T,W,Y,V;preferably X286N,C,Q,I,L,M,P,T,V,Y,F;
X287R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X288A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X289A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
15 X292A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X295A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X296A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X304C,M;
X307A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
20 X312A,N,C,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
X313A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X320R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X322R,N,D,C,Q,E,G,H,L,K,M,F,P,S,T,W,Y,V;
X323A,R,N,D,C,Q,E,G,I,L,K,M,F,P,S,T,W,Y,V;
25 X325A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X326A,R,N,C,Q,E,G,M,P,S,T,W;
X327A,R,C,G,H,I,L,K,M,P,S,T,W,Y,V;
X329A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,T,W,Y,V;
X331N,D,C,Q,E,G,H,I,L,M,F,P,S,T,W,Y,V;
30 X339A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X343A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,Y,V;
X344A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X346A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X347A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
35 X349R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
X350A,R,N,C,Q,G,H,I,L,K,M,F,P,S,T,Y,V;

- X359R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X360N,D,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 X369A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X377A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 5 X380A,R,N,D,C,Q,E,G,H,K,P,S,W,Y,V;
 X387A,R,N,D,C,Q,E,G,H,L,K,M,P,S,T,W,Y,V;
 X409N,C,Q,E,G,H,M,P,S,T,W,Y,V;
 X410A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X411A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 10 X412R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X423A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X424A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X426A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X427A,R,N,D,C,Q,E,G,H,K,M,P,S,T,Y,V;
 15 X428A,N,D,Q,E,G,H,I,M,F,P,S,W,Y,V;
 X429A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X430A,R,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X438C,M;
 X440R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 20 X441A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X449A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X462A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X472A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 X477A,R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 25 X479A,R,N,D,Q,E,G,H,I,L,K,M,F,P,S,W,Y,V;
 X480A,R,D,C,G,K,M,P,W,Y;
 X481A,R,N,D,C,Q,E,G,H,K,M,P,S,T,Y,V

wherein

- (a) the variant has alpha-amylase activity and (b) each position corresponds to a position of
 30 the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid
 sequence shown in SEQ ID NO: 8 (*Bacillus licheniformis* alpha-amylase).

4. A variant of a parent Termamyl-like alpha-amylase, comprising an alteration at one or more
 positions selected from the group of:

- 35 A1 insertion;
 L3 insertion;

N4 insertion;
N17 insertion;
D18 insertion;
Q20 insertion;
5 R23 insertion;
R24 insertion;
D28 insertion;
Y56 insertion;
L61 insertion or deletion;
10 Y62 insertion;
F67 insertion or deletion;
H68 insertion;
K80 insertion or deletion;
G81 insertion or deletion;
15 Q84 insertion;
S85 insertion;
H91 insertion or deletion;
S92 insertion or deletion;
K106 insertion or deletion;
20 D110 insertion or deletion;
D114 deletion;
E119 insertion or deletion;
D121 insertion;
P122 insertion;
25 A123 insertion;
D124 insertion;
R125 insertion;
N126 insertion;
R127 insertion;
30 I129 insertion;
G131 insertion;
L134 insertion;
K136 insertion;
N172 insertion;
35 E185 insertion;
L196 insertion or deletion;

P206 insertion or deletion;
T217 insertion;
W218 insertion;
D231 insertion or deletion;
5 A232 insertion or deletion;
H235 insertion or deletion;
N246 insertion;
H247 insertion;
R249 insertion;
10 K251 insertion;
F257 insertion or deletion;
N278 insertion;
G310 insertion or deletion;
H316 insertion;
15 P317 insertion;
D328 insertion or deletion;
G332 insertion or deletion;
E355 insertion or deletion;
Y358 insertion;
20 Y363 insertion;
Y367 insertion;
K370 insertion;
S373 insertion;
R375 insertion;
25 E376 insertion;
K381 insertion;
H382 insertion;
R391 insertion or deletion;
Y396 insertion;
30 R413 insertion or deletion;
E414 insertion or deletion;
G415 insertion or deletion;
D416 insertion;
S417 insertion;
35 S418 insertion;
V419 insertion;

- A420 insertion;
N421 insertion;
S422 insertion or deletion;
Y439 insertion;
- 5 A445 insertion or deletion;
G446 insertion or deletion;
T448 insertion or deletion;
H450 insertion;
G454 insertion or deletion;
- 10 N455 insertion;
E458 insertion;
P459 insertion;
V460 insertion;
V461 insertion;
- 15 N463 insertion;
S464 insertion;
E465 insertion;
W467 insertion;
wherein
- 20 (a) the alteration(s) are independently (as specified above):
(i) an insertion of an amino acid downstream of the amino acid which occupies the position, or
(ii) a deletion of the amino acid which occupies the position,
(b) the variant has alpha-amylase activity and (c) each position corresponds to a position of the
25 amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8 (*Bacillus licheniformis* alpha-amylase).
5. A variant of a parent Termamyl-like alpha-amylase, comprising an alteration at one or more positions selected from the group of:
- 30 L7 insertion or deletion;
M8 insertion;
Y10 insertion;
F11 insertion;
E12 insertion or deletion;
- 35 M15 insertion;
G19 insertion;

H21 insertion;
W22 insertion;
L25 insertion;
V40 insertion or deletion;
5 W41 insertion;
P43 insertion or deletion;
P44 insertion or deletion;
Y46 insertion;
G55 insertion;
10 Y59 insertion;
Y77 insertion;
G78 insertion or deletion;
L90 insertion or deletion;
I95 insertion;
15 V97 insertion;
Y98 insertion;
G99 insertion;
D100 insertion;
V101 insertion;
20 V102 insertion;
H105 insertion or deletion;
A109 insertion or deletion;
V115 insertion or deletion;
V118 insertion or deletion;
25 I135 insertion;
T139 insertion or deletion;
F141 insertion or deletion;
Y195 insertion;
V208 insertion or deletion;
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Y219 insertion;
I236 insertion or deletion;
F238 insertion or deletion;
F240 insertion or deletion;
35 W244 insertion;
V248 insertion;

- M256 insertion;
T258 insertion or deletion;
V259 insertion or deletion;
V312 insertion or deletion;
5 V313 insertion or deletion;
S320 insertion;
T322 insertion or deletion;
F323 insertion or deletion;
D325 insertion or deletion;
10 N326 insertion;
H327 insertion or deletion;
Q330 insertion or deletion;
P331 insertion or deletion;
Y348 insertion;
15 A349 insertion or deletion;
F350 insertion or deletion;
P359 insertion or deletion;
Q360 insertion;
D365 insertion or deletion;
20 M366 insertion;
T369 insertion;
I377 insertion;
I384 insertion or deletion;
L388 insertion or deletion;
25 G423 insertion or deletion;
L424 insertion or deletion;
M438 insertion;
G441 insertion or deletion;
W449 insertion;
30 I462 insertion;
I479 insertion or deletion;
Y480 insertion;
V481 insertion or deletion;
wherein
35 (a) the alteration(s) are independently (as specified above):
(i) an insertion of an amino acid downstream of the amino acid which occupies the

position, or

(ii) a deletion of the amino acid which occupies the position,

(b) the variant has alpha-amylase activity and (c) each position corresponds to a position of the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid

5 sequence shown in SEQ ID NO: 8 (*Bacillus licheniformis* alpha-amylase).

6. The variant of any of claims 1-5, wherein the parent Termamyl-like alpha-amylase is derived from a strain of *B. licheniformis* (SEQ ID NO: 8), *B. amyloliquefaciens* (SEQ ID NO: 10), *B. stearothermophilus* (SEQ ID NO: 6), *Bacillus* sp. (SEQ ID NO: 12 (AA560)), *Bacillus* sp. (SEQ ID NO: 2 (SP690)); *Bacillus* sp. (SEQ ID NO: 4 (SP722)); *Bacillus* sp. #707 alpha-amylase (SEQ ID NO: 13); KSM-AP1378.

7. The variant of any of claims 1 to 6, wherein the parent Termamyl-like alpha-amylase has an amino acid sequence which has a degree of identity to SEQ ID NO: 8 of at least 60%, preferably 15 70%, more preferably at least 80%, even more preferably at least about 90%, even more preferably at least 95%, even more preferably at least 96%, even more preferably at least 97%, even more preferably at least 98%, and even more preferably at least 99%.

8. The variant of any of claims 1-7, wherein the parent Termamyl-like alpha-amylase is encoded 20 by a nucleic acid sequence (DNA), which hybridizes under low, preferably medium, preferred high stringency conditions, with the nucleic acid sequence of SEQ ID NO: 7.

9. The variant of claims 1-8, which variant has alpha-amylase activity and exhibits an alteration in at least one of the following properties relative to said parent alpha-amylase: altered substrate 25 specificity, substrate binding, substrate cleavage pattern, thermal stability, pH activity profile, pH stability profile, stability towards oxidation, Ca^{2+} dependency, reduced and increased pI and improved wash performance, specific activity, stability under high temperature and/or low pH conditions, in particular at low calcium concentrations and/or in particular at high temperatures from 70-120°C and/or low pH in the range from pH 4-6

30

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

11. A recombinant expression vector which carries a nucleic acid sequence (DNA) construct 35 according to claim 10.

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

13. A cell according to claim 12, which is a microorganism, preferably a bacterium or a fungus,
5 in particular a gram-positive bacterium, such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus* or *Bacillus thuringiensis*.

14. A composition comprising an alpha-amylase variant of any of claims 1-9.

10

15. The composition of claim 14, wherein the composition further comprises glucoamylase, pullulanase and/or phytase.

16. A detergent composition comprising an alpha-amylase variant according to any of claims 1-
15 9.

17. A detergent composition of claim 16, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme, glucoamylase, maltogenic amylase, CGTase, mannanase, cutinase, laccase and/or a cellulase.

20

18. Use of an alpha-amylase variant according to any of claims 1-9 or a composition according to claim 15 for starch liquefaction, in particular for syrup or ethanol production.

.

19. Use of an alpha-amylase variant according to any one of claims 1-9 or a composition
25 according to claim 15 for washing and/or dishwashing.

20. Use of an alpha-amylase variant of any one of claims 1-9 or a composition according to claim 15 for textile desizing.

	1				50
1	HHNGTNGTMM	QYFEWHL PND	GNHWNRLRDD	ASNLNRNGIT	AIWIPPAWKG
2	HHNGTNGTMM	QYFEWYLPND	GNHWNRLRDD	AANLKSKGIT	AVWIPPAWKG
3	 VNGTLM	QYFEWYTPND	GQHWKRLQND	AEHLSDIGIT	AVWIPPAYKG
4	. . ANLNGTLM	QYFEWYMPND	GQHWRR LQND	SAYLAEHGIT	AVWIPPAYKG
5	. AAPFNGTMM	QYFEWYLPDD	GTLWTKVANE	ANNLSSLGIT	ALWLPPAYKG
	51				100
1	TSQNDVGYGA	YDLYDLGEFN	QKGTVRTKYG	TRSQLESaih	ALKNNGVQVY
2	TSQNDVGYGA	YDLYDLGEFN	QKGTVRTKYG	TRNQLQAAVT	SLKNNGIQVY
3	LSQSDNGYGP	YDLYDLGEFQ	QKGTVRTKYG	TKSELQDAIG	SLHSRNVQVY
4	TSQADVGYGA	YDLYDLGEFH	QKGTVRTKYG	TKGELQSAIK	SLHSRDIN VY
5	TSRSDVGYGV	YDLYDLGEFN	QKGTVRTKYG	TKAQYLQAIQ	AAHAAGMQVY
	101				150
1	GDVVMNHKGG	ADATENVLAV	EVNPNNRNQE	ISGDYTIEAW	TKFDFPGRGN
2	GDVVMNHKGG	ADGTEIVNAV	EVNRSNRNQE	TSGEYAIEAW	TKFDFPGRGN
3	GDVVLNHKAG	ADATEDVTAV	EVNPNARNQE	TSEFYQIKAW	TDFRFPGRGN
4	GDVVINHKGG	ADATEDVTAV	EVDPADRNRV	ISGEHLIKAW	THFHFPGRGS
5	ADVVFDPKGG	ADGTEWVDAV	EVNPSDRNQE	ISGTYQIQAW	TKFDFPGRGN
	151				200
1	TYSDFKWRWY	HFDGVDWDQS	RQFQNR IYKF	RGDGKA WDWE	VDSENGNYDY
2	NHSSF KWRWY	HFDGTDWDQS	RQLQNKIYKF	RGTGKA WDWE	VDTENGN YDY
3	TYSDFKWHWY	HFDGADWDES	RKI .SRIFKF	RGEGKA WDWE	VVSENGNYDY
4	TYSDFKWHWY	HFDGTDWDES	RKL .NRIYKF	. .QGKA WDWE	VSNENGNYDY
5	TYSSFKWRWY	HFDGVDWDES	RKL .SRIYKF	RGIGKA WDWE	VDTENGN YDY

Fig. 1

	201				250
1	LMYADVDMDH	PEVVNELRRW	GEWYTNTLNL	DGFRIDAVKH	IKYSFTRDWL
2	LMYADVDMDH	PEVIHELNRW	GVWYTNTLNL	DGFRIDAVKH	IKYSFTRDWL
3	LMYADVDDYDH	PDVVAETKKW	GIWYANELSL	DGFRIDAACH	IKFSFLRDWV
4	LMYADIDYDH	PDVAAEIKRW	GTWYANELQL	DGFRIDAVKH	IKFSFLRDWV
5	LMYADLDMDH	PEVVTELKNW	GKWYVNTTNI	DGFRIDAVKH	IKFSFFPDWL
	251				300
1	THVRNATGKE	MFAVAEFWKN	DLGALENYLN	KTNNWNHVSVD	VPLHYNLYNA
2	THVRNTTGKP	MFAVAEFWKN	DLGAIENYLN	KTNNWNHSAFD	VPLHYNLYNA
3	QAVRQATGKE	MFTVAEYWQN	NAGKLENYLN	KTNNWNHVSVD	VPLHFNQAA
4	NHVREKTGKE	MFTVAEYWQN	DLGALENYLN	KTNNWNHVSVD	VPLHYQFHAA
5	SYVRSQTGKP	LFTVGEYWSY	DINKLHNYIT	KTNNWNHVSVD	APLHNKFYTA
	301				350
1	SNSGGNYDMA	KLLNGTVVQK	HPMHAVTFVD	NHDSQPGESL	ESFVQEWFKP
2	SNSGGYYDMR	NILNGSVVQK	HPTHAVTFVD	NHDSQPGESL	ESFVQQWFKP
3	SSQGGGYDMR	RLLDGTVVSR	HPEKAVTFVE	NHDTQPGQSL	ESTVQTFWFKP
4	STQGGGYDMR	KLLNGTVVSK	HPLKSVTFVD	NHDTQPGQSL	ESTVQTFWFKP
5	SKSGGAFDMR	TLMTNTLMKD	QPTLAVTFVD	NHDTQPGQSL	QSWVDPWFKP
	351				400
1	LAYALILTRE	QGYPSVFYGD	YYGIPTHS..	.VPAMKAKID	PILEARQNFA
2	LAYALVLTRE	QGYPSVFYGD	YYGIPTHG..	.VPAMKSKID	PLLQARQTFA
3	LAYAFILTRE	SGYPQVFYGD	MYGTKGTSPK	EIPSLKDNIE	PILKARKEYA
4	LAYAFILTRE	SGYPQVFYGD	MYGTKGDSQR	EIPALKHKIE	PILKARKQYA
5	LAYAFILTRQ	EGYPCVFYGD	YYGIPQYN..	.IPSLKSKID	PLLIARRDYA
	401				450
1	YGTQHDYFDH	HNIIGWTREG	NTTHPNSGLA	TIMSDGPGGE	KWMYVGQNKA
2	YGTQHDYFDH	HDIIGWTREG	NSSHPSNSGLA	TIMSDGPGGN	KWMYVGKNKA
3	YGPQHDYIDH	PDVIGWTREG	DSSAAKSGLA	ALITDGPGGG	KRMYAGLKNA
4	YGAQHDYFDH	HDIIGWTREG	DSSVANSGLA	ALITDGPGGG	KRMYVGRQNA
5	YGTQHDYLDH	SDIIGWTREG	GTEKPGSGLA	ALITDGPGGG	KWMYVGKQHA

Fig. 1 (continued)

	451					500
1	GQVWHDITGN	KPGTVTINAD	GWANFSVNGG	SVSIWVKR..		
2	GQVWRDITGN	RTGTVTINAD	GWGNFSVNGG	SVSVWVKQ..		
3	GETWYDITGN	RSDTVKIGSD	GWGEFHVNDG	SVSIYVQ...		
4	GETWHDITGN	RSEPVVINSE	GWGEFHVNGG	SVSIYVQR..		
5	GKVFYDLTGN	RSDTVTINS	DGWGEFKVNGG	SVSVWVPRKT	TVSTIARPIT	
	501		519			
1						
2						
3						
4						
5	TRPWTGEFVR	WTEPRLVAW				

Fig. 1 (continued)

10182.ST25
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<160> 13

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Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp	
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Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr	
50 55 60	
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Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly	
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aca cgc aac cag cta cag gct gcg gtg acc tct tta aaa aat aac ggc	288
Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly	
85 90 95	

10182.ST25

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ggt Gly	acg Thr	gaa Glu 115	att Ile	gta Val	aat Asn	gcg Ala	gta Val 120	gaa Glu	gtg Val	aat Asn	cgg Arg	agc Ser 125	aac Asn	cga Arg	aac Asn	384
cag Gln	gaa Glu 130	acc Thr	tca Ser	gga Gly	gag Glu	tat Tyr 135	gca Ala	ata Ile	gaa Glu	gcg Ala	tgg Trp 140	aca Thr	aag Lys	ttt Phe	gat Asp	432
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ggt Gly 305	tat Tyr	tat Tyr	gat Asp	atg Met	aga Arg 310	aat Asn	att Ile	tta Leu	aat Asn	ggt Gly 315	tct Ser	gtg Val	gtg Val	caa Gln	aaa Lys 320	960
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10182.ST25

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Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr	
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10182.ST25

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 195 200 205
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 Tyr Ala Leu Val Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
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Page 5

6/28

10182.ST25															
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Val	Gln	Val	Tyr	Gly	Asp	Val	Val	Met	Asn	His	Lys	Gly	Gly	Ala	Asp
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Ile	Lys	Tyr	Ser	Phe	Thr	Arg	Asp	Trp	Leu	Thr	His	Val	Arg	Asn	Ala
				245					250					255	
acg	gga	aaa	gaa	atg	ttt	gct	gtt	gct	gaa	ttt	tgg	aaa	aat	gat	tta
Thr	Gly	Lys	Glu	Met	Phe	Ala	Val	Ala	Glu	Phe	Trp	Lys	Asn	Asp	Leu
			260					265					270		
ggt	gcc	ttg	gag	aac	tat	tta	aat	aaa	aca	aac	tgg	aat	cat	tct	gtc
Gly	Ala	Leu	Glu	Asn	Tyr	Leu	Asn	Lys	Thr	Asn	Trp	Asn	His	Ser	Val
		275					280					285			
ttt	gat	gtc	ccc	ctt	cat	tat	aat	ctt	tat	aac	gcg	tca	aat	agt	gga
Phe	Asp	Val	Pro	Leu	His	Tyr	Asn	Leu	Tyr	Asn	Ala	Ser	Asn	Ser	Gly
	290					295					300				
ggc	aac	tat	gac	atg	gca	aaa	ctt	ctt	aat	gga	acg	gtt	gtt	caa	aag
Gly	Asn	Tyr	Asp	Met	Ala	Lys	Leu	Leu	Asn	Gly	Thr	Val	Val	Gln	Lys
305					310					315					320
cat	cca	atg	cat	gcc	gta	act	ttt	gtg	gat	aat	cac	gat	tct	caa	cct

10182.ST25
 His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
 325 330 335
 ggg gaa tca tta gaa tca ttt gta caa gaa tgg ttt aag cca ctt gct 1056
 Gly Glu Ser Leu Glu Ser Phe Val Gln Glu Trp Phe Lys Pro Leu Ala
 340 345 350
 tat gcg ctt att tta aca aga gaa caa ggc tat ccc tct gtc ttc tat 1104
 Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
 355 360 365
 ggt gac tac tat gga att cca aca cat agt gtc cca gca atg aaa gcc 1152
 Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala
 370 375 380
 aag att gat cca atc tta gag gcg cgt caa aat ttt gca tat gga aca 1200
 Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Asn Phe Ala Tyr Gly Thr
 385 390 400
 caa cat gat tat ttt gac cat cat aat ata atc gga tgg aca cgt gaa 1248
 Gln His Asp Tyr Phe Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
 405 410 415
 gga aat acc acg cat ccc aat tca gga ctt gcg act atc atg tcg gat 1296
 Gly Asn Thr Thr His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
 420 425 430
 ggg cca ggg gga gag aaa tgg atg tac gta ggg caa aat aaa gca ggt 1344
 Gly Pro Gly Gly Glu Lys Trp Met Tyr Val Gly Gln Asn Lys Ala Gly
 435 440 445
 caa gtt tgg cat gac ata act gga aat aaa cca gga aca gtt acg atc 1392
 Gln Val Trp His Asp Ile Thr Gly Asn Lys Pro Gly Thr Val Thr Ile
 450 455 460
 aat gca gat gga tgg gct aat ttt tca gta aat gga gga tct gtt tcc 1440
 Asn Ala Asp Gly Trp Ala Asn Phe Ser Val Asn Gly Gly Ser Val Ser
 465 470 475 480
 att tgg gtg aaa cga
 Ile Trp Val Lys Arg 485 1455

<210> 4

<211> 485

<212> PRT

<213> Bacillus sp.

<400> 4

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 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

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
 50 55 60 10182.ST25

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

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

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

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

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

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

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

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

10182.ST25

Ile Trp Val Lys Arg
485

<213> *Bacillus stearothermophilus*

<223> BSG

gcc gca ccg ttt aac ggc acc atg atg cag tat ttt gaa tgg tac ttg
Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu
1 5 10 15

10/28

10182.ST25

ccg	gat	gat	ggc	acg	tta	tgg	acc	aaa	gtg	gcc	aat	gaa	gcc	aac	aac	96
Pro	Asp	Asp	Gly 20	Thr	Leu	Trp	Thr	Lys 25	Val	Ala	Asn	Glu	Ala 30	Asn	Asn	
tta	tcc	agc	ctt	ggc	atc	acc	gct	ctt	tgg	ctg	ccg	ccc	gct	tac	aaa	144
Leu	Ser	Ser 35	Leu	Gly	Ile	Thr	Ala 40	Leu	Trp	Leu	Pro	Pro 45	Ala	Tyr	Lys	
gga	aca	agc	cgc	agc	gac	gta	ggg	tac	gga	gta	tac	gac	ttg	tat	gac	192
Gly	Thr 50	Ser	Arg	Ser	Asp	Val 55	Gly	Tyr	Gly	Val	Tyr 60	Asp	Leu	Tyr	Asp	
ctc	ggc	gaa	ttc	aat	caa	aaa	ggg	acc	gtc	cgc	aca	aaa	tac	gga	aca	240
Leu 65	Gly	Glu	Phe	Asn	Gln 70	Lys	Gly	Thr	Val	Arg 75	Thr	Lys	Tyr	Gly	Thr 80	
aaa	gct	caa	tat	ctt	caa	gcc	att	caa	gcc	gcc	cac	gcc	gct	gga	atg	288
Lys	Ala	Gln	Tyr	Leu 85	Gln	Ala	Ile	Gln	Ala 90	Ala	His	Ala	Ala	Gly 95	Met	
caa	gtg	tac	gcc	gat	gtc	gtg	ttc	gac	cat	aaa	ggc	ggc	gct	gac	ggc	336
Gln	Val	Tyr	Ala 100	Asp	Val	Val	Phe	Asp 105	His	Lys	Gly	Gly	Ala 110	Asp	Gly	
acg	gaa	tgg	gtg	gac	gcc	gtc	gaa	gtc	aat	ccg	tcc	gac	cgc	aac	caa	384
Thr	Glu	Trp 115	Val	Asp	Ala	Val	Glu 120	Val	Asn	Pro	Ser	Asp 125	Arg	Asn	Gln	
gaa	atc	tcg	ggc	acc	tat	caa	atc	caa	gca	tgg	acg	aaa	ttt	gat	ttt	432
Glu	Ile 130	Ser	Gly	Thr	Tyr	Gln 135	Ile	Gln	Ala	Trp	Thr 140	Lys	Phe	Asp	Phe	
ccc	ggg	cgg	ggc	aac	acc	tac	tcc	agc	ttt	aag	tgg	cgc	tgg	tac	cat	480
Pro 145	Gly	Arg	Gly	Asn	Thr 150	Tyr	Ser	Ser	Phe	Lys 155	Trp	Arg	Trp	Tyr	His 160	
ttt	gac	ggc	gtt	gat	tgg	gac	gaa	agc	cga	aaa	ttg	agc	cgc	att	tac	528
Phe	Asp	Gly	Val	Asp 165	Trp	Asp	Glu	Ser	Arg 170	Lys	Leu	Ser	Arg	Ile 175	Tyr	
aaa	ttc	cgc	ggc	atc	ggc	aaa	gcg	tgg	gat	tgg	gaa	gta	gac	acg	gaa	576
Lys	Phe	Arg	Gly 180	Ile	Gly	Lys	Ala 185	Trp	Asp	Trp	Glu	Val	Asp 190	Thr	Glu	
aac	gga	aac	tat	gac	tac	tta	atg	tat	gcc	gac	ctt	gat	atg	gat	cat	624
Asn	Gly	Asn 195	Tyr	Asp	Tyr	Leu	Met 200	Tyr	Ala	Asp	Leu	Asp 205	Met	Asp	His	
ccc	gaa	gtc	gtg	acc	gag	ctg	aaa	aac	tgg	ggg	aaa	tgg	tat	gtc	aac	672
Pro 210	Glu	Val	Val	Thr	Glu	Leu 215	Lys	Asn	Trp	Gly	Lys 220	Trp	Tyr	Val	Asn	
aca	acg	aac	att	gat	ggg	ttc	cgg	ctt	gat	gcc	gtc	aag	cat	att	aag	720
Thr 225	Thr	Asn	Ile	Asp	Gly 230	Phe	Arg	Leu	Asp	Ala 235	Val	Lys	His	Ile	Lys 240	
ttc	agt	ttt	ttt	cct	gat	tgg	ttg	tcg	tat	gtg	cgt	tct	cag	act	ggc	768
Phe	Ser	Phe	Phe	Pro 245	Asp	Trp	Leu	Ser	Tyr 250	Val	Arg	Ser	Gln	Thr 255	Gly	
aag	ccg	cta	ttt	acc	gtc	ggg	gaa	tat	tgg	agc	tat	gac	atc	aac	aag	816
Lys	Pro	Leu	Phe 260	Thr	Val	Gly	Glu	Tyr 265	Trp	Ser	Tyr	Asp	Ile 270	Asn	Lys	
ttg	cac	aat	tac	att	acg	aaa	aca	gac	gga	acg	atg	tct	ttg	ttt	gat	864
Leu	His	Asn 275	Tyr	Ile	Thr	Lys	Thr 280	Asp	Gly	Thr	Met	Ser 285	Leu	Phe	Asp	

11/28

10182.ST25

gcc	ccg	tta	cac	aac	aaa	ttt	tat	acc	gct	tcc	aaa	tca	ggg	ggc	gca	912
Ala	Pro	Leu	His	Asn	Lys	Phe	Tyr	Thr	Ala	Ser	Lys	Ser	Gly	Gly	Ala	
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ttt	gat	atg	cgc	acg	tta	atg	acc	aat	act	ctc	atg	aaa	gat	caa	ccg	960
Phe	Asp	Met	Arg	Thr	Leu	Met	Thr	Asn	Thr	Leu	Met	Lys	Asp	Gln	Pro	
305					310					315					320	
aca	ttg	gcc	gtc	acc	ttc	gtt	gat	aat	cat	gac	acc	gaa	ccc	ggc	caa	1008
Thr	Leu	Ala	Val	Thr	Phe	Val	Asp	Asn	His	Asp	Thr	Glu	Pro	Gly	Gln	
				325					330					335		
gcg	ctg	cag	tca	tgg	gtc	gac	cca	tgg	ttc	aaa	ccg	ttg	gct	tac	gcc	1056
Ala	Leu	Gln	Ser	Trp	Val	Asp	Pro	Trp	Phe	Lys	Pro	Leu	Ala	Tyr	Ala	
			340					345					350			
ttt	att	cta	act	cgg	cag	gaa	gga	tac	ccg	tgc	gtc	ttt	tat	ggg	gac	1104
Phe	Ile	Leu	Thr	Arg	Gln	Glu	Gly	Tyr	Pro	Cys	Val	Phe	Tyr	Gly	Asp	
		355					360					365				
tat	tat	ggc	att	cca	caa	tat	aac	att	cct	tcg	ctg	aaa	agc	aaa	atc	1152
Tyr	Tyr	Gly	Ile	Pro	Gln	Tyr	Asn	Ile	Pro	Ser	Leu	Lys	Ser	Lys	Ile	
	370					375					380					
gat	ccg	ctc	ctc	atc	gcg	cgc	agg	gat	tat	gct	tac	gga	acg	caa	cat	1200
Asp	Pro	Leu	Leu	Ile	Ala	Arg	Arg	Asp	Tyr	Ala	Tyr	Gly	Thr	Gln	His	
385					390					395					400	
gat	tat	ctt	gat	cac	tcc	gac	atc	atc	ggg	tgg	aca	agg	gaa	ggg	ggc	1248
Asp	Tyr	Leu	Asp	His	Ser	Asp	Ile	Ile	Gly	Trp	Thr	Arg	Glu	Gly	Gly	
				405					410					415		
act	gaa	aaa	cca	gga	tcc	gga	ctg	gcc	gca	ctg	atc	acc	gat	ggg	ccg	1296
Thr	Glu	Lys	Pro	Gly	Ser	Gly	Leu	Ala	Ala	Leu	Ile	Thr	Asp	Gly	Pro	
			420					425					430			
gga	gga	agc	aaa	tgg	atg	tac	gtt	ggc	aaa	caa	cac	gct	gga	aaa	gtg	1344
Gly	Gly	Ser	Lys	Trp	Met	Tyr	Val	Gly	Lys	Gln	His	Ala	Gly	Lys	Val	
		435					440					445				
ttc	tat	gac	ctt	acc	ggc	aac	cgg	agt	gac	acc	gtc	acc	atc	aac	agt	1392
Phe	Tyr	Asp	Leu	Thr	Gly	Asn	Arg	Ser	Asp	Thr	Val	Thr	Ile	Asn	Ser	
	450					455					460					
gat	gga	tgg	ggg	gaa	ttc	aaa	gtc	aat	ggc	ggg	tcg	gtt	tcg	gtt	tgg	1440
Asp	Gly	Trp	Gly	Glu	Phe	Lys	Val	Asn	Gly	Gly	Ser	Val	Ser	Val	Trp	
465					470				475						480	
gtt	cct	aga	aaa	acg	acc	gtt	tct	acc	atc	gct	cgg	ccg	atc	aca	acc	1488
Val	Pro	Arg	Lys	Thr	Thr	Val	Ser	Thr	Ile	Ala	Arg	Pro	Ile	Thr	Thr	
				485					490					495		
cga	ccg	tgg	act	ggg	gaa	ttc	gtc	cgt	tgg	acc	gaa	cca	cgg	ttg	gtg	1536
Arg	Pro	Trp	Thr	Gly	Glu	Phe	Val	Arg	Trp	Thr	Glu	Pro	Arg	Leu	Val	
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gca	tgg	cct	tga													1548
Ala	Trp	Pro														
		515														

<210> 6

<211> 515

<212> PRT

12/28

10182.ST25

<213> Bacillus stearothermophilus

<400> 6

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 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 Thr 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 Asn 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
 225 230 235 240
 Phe Ser Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln Thr Gly
 245 250 255

Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys
 260 265 270
 Leu His Asn Tyr Ile Thr Lys Thr Asp 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 Ala
 290 295 300
 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
 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
 355 360 365
 Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile
 370 375 380
 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 Gly
 405 410 415
 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
 435 440 445
 Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser
 450 455 460
 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 Arg Pro Ile Thr Thr
 485 490 495
 Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro Arg Leu Val
 500 505 510
 Ala Trp Pro
 515

<210> 7

<211> 1920

<212> DNA

<213> Bacillus licheniformis

<220>

<221> CDS

<222> (421)..(1872)

<223> TERMAMYL

<400> 7

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agattattaa aaagctgaaa gcaaaaggct atcaattggg aactgtatct cagcttgaag	180
aagtgaagaa gcagagaggc tattgaataa atgagtagaa gcgccatata ggcgccttttc	240
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tcatatgttt cacattgaaa ggggaggaga atcatgaaac aacaaaaacg gctttacgcc	360
cgattgctga cgctgttatt tgcgctcatc ttcttgctgc ctcatctctgc agcagcggcg	420
gca aat ctt aat ggg acg ctg atg cag tat ttt gaa tgg tac atg ccc	468
Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro	
1 5 10 15	
aat gac ggc caa cat tgg agg cgt ttg caa aac gac tcg gca tat ttg	516
Asn Asp Gly Gln His Trp Arg Arg Leu Gln Asn Asp Ser Ala Tyr Leu	
20 25 30	
gct gaa cac ggt att act gcc gtc tgg att ccc ccg gca tat aag gga	564
Ala Glu His Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly	
35 40 45	
acg agc caa gcg gat gtg ggc tac ggt gct tac gac ctt tat gat tta	612
Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu	
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ggg gag ttt cat caa aaa ggg acg gtt cgg aca aag tac ggc aca aaa	660
Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys	
65 70 75 80	
gga gag ctg caa tct gcg atc aaa agt ctt cat tcc cgc gac att aac	708
Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn	
85 90 95	
gtt tac ggg gat gtg gtc atc aac cac aaa ggc ggc gct gat gcg acc	756
Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr	
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gaa gat gta acc gcg gtt gaa gtc gat ccc gct gac cgc aac cgc gta	804
Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val	
115 120 125	

10182.ST25

att Ile	tca Ser 130	gga Gly	gaa Glu	cac His	cta Leu	att Ile 135	aaa Lys	gcc Ala	tgg Trp	aca Thr	cat His 140	ttt Phe	cat His	ttt Phe	ccg Pro	852
ggg Gly 145	cgc Arg	ggc Gly	agc Ser	aca Thr	tac Tyr 150	agc Ser	gat Asp	ttt Phe	aaa Lys	tgg Trp 155	cat His	tgg Trp	tac Tyr	cat His	ttt Phe 160	900
gac Asp	gga Gly	acc Thr	gat Asp	tgg Trp 165	gac Asp	gag Glu	tcc Ser	cga Arg	aag Lys 170	ctg Leu	aac Asn	cgc Arg	atc Ile	tat Tyr 175	aag Lys	948
ttt Phe	caa Gln	gga Gly	aag Lys 180	gct Ala	tgg Trp	gat Asp	tgg Trp	gaa Glu 185	gtt Val	tcc Ser	aat Asn	gaa Glu	aac Asn 190	ggc Gly	aac Asn	996
tat Tyr	gat Asp	tat Tyr 195	ttg Leu	atg Met	tat Tyr	gcc Ala	gac Asp 200	atc Ile	gat Asp	tat Tyr	gac Asp	cat His 205	cct Pro	gat Asp	gtc Val	1044
gca Ala 210	gca Ala	gaa Glu	att Ile	aag Lys	aga Arg	tgg Trp 215	ggc Gly	act Thr	tgg Trp	tat Tyr	gcc Ala 220	aat Asn	gaa Glu	ctg Leu	caa Gln	1092
ttg Leu 225	gac Asp	ggt Gly	ttc Phe	cgt Arg	ctt Leu 230	gat Asp	gct Ala	gtc Val	aaa Lys	cac His 235	att Ile	aaa Lys	ttt Phe	tct Ser	ttt Phe 240	1140
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tat Tyr	ttg Leu	aac Asn 275	aaa Lys	aca Thr	aat Asn	ttt Phe	aat Asn 280	cat His	tca Ser	gtg Val	ttt Phe	gac Asp 285	gtg Val	ccg Pro	ctt Leu	1284
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agg Arg 305	aaa Lys	ttg Leu	ctg Leu	aac Asn	ggt Gly 310	acg Thr	gtc Val	gtt Val	tcc Ser	aag Lys 315	cat His	ccg Pro	ttg Leu	aaa Lys	tcg Ser 320	1380
gtt Val	aca Thr	ttt Phe	gtc Val	gat Asp 325	aac Asn	cat His	gat Asp	aca Thr	cag Gln 330	ccg Pro	ggg Gly	caa Gln	tcg Ser	ctt Leu 335	gag Glu	1428
tcg Ser	act Thr	gtc Val	caa Gln 340	aca Thr	tgg Trp	ttt Phe	aag Lys	ccg Pro 345	ctt Leu	gct Ala	tac Tyr	gct Ala	ttt Phe 350	att Ile	ctc Leu	1476
aca Thr	agg Arg	gaa Glu 355	tct Ser	gga Gly	tac Tyr	cct Pro	cag Gln 360	gtt Val	ttc Phe	tac Tyr	ggg Gly	gat Asp 365	atg Met	tac Tyr	ggg Gly	1524
acg Thr	aaa Lys 370	gga Gly	gac Asp	tcc Ser	cag Gln	cgc Arg 375	gaa Glu	att Ile	cct Pro	gcc Ala	ttg Leu 380	aaa Lys	cac His	aaa Lys	att Ile	1572
gaa Glu 385	ccg Pro	atc Ile	tta Leu	aaa Lys	gcg Ala 390	aga Arg	aaa Lys	cag Gln	tat Tyr	gcg Ala 395	tac Tyr	gga Gly	gca Ala	cag Gln	cat His 400	1620

16/28

10182.ST25

gat tat ttc gac cac cat gac att gtc ggc tgg aca agg gaa ggc gac 1668
 Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp
 405 410 415

agc tcg gtt gca aat tca ggt ttg gcg gca tta ata aca gac gga ccc 1716
 Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
 420 425 430

ggt ggg gca aag cga atg tat gtc ggc cgg caa aac gcc ggt gag aca 1764
 Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr
 435 440 445

tgg cat gac att acc gga aac cgt tcg gag ccg gtt gtc atc aat tcg 1812
 Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser
 450 455 460

gaa ggc tgg gga gag ttt cac gta aac ggc ggg tcg gtt tca att tat 1860
 Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr
 465 470 475 480

ggt caa aga tag aagagcagag aggacggatt tcctgaagga aatccgtttt 1912
 Val Gln Arg

tttatttt 1920

<210> 8

<211> 483

<212> PRT

<213> Bacillus licheniformis

<400> 8

Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro
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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

10182.ST25

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

Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met
245 250 255

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

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

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

Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile
370 375 380

10182.ST25

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

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

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

Val Gln Arg

<210> 9

<211> 2084

<212> DNA

<213> Bacillus amyloliquefaciens

<220>

<221> CDS

<222> (343)..(1794)

<223> BAN

<400> 9

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atcagacagg gtatttttta tgctgtccag actgtccgct gtgtaaaaat aaggaataaa 180

gggggggttg tattatttta ctgatatgta aaatataatt tgtataagaa aatgagaggg 240

agaggaaaca tgattcaaaa acgaaagcgg acagtttcgt tcagacttgt gcttatgtgc 300

acgctgttat ttgtcagttt gccgattaca aaaacatcag cc gta aat ggc acg 354

Val Asn Gly Thr
1

ctg atg cag tat ttt gaa tgg tat acg ccg aac gac ggc cag cat tgg 402

Leu Met Gln Tyr Phe Glu Trp Tyr Thr Pro Asn Asp Gly Gln His Trp 20

aaa cga ttg cag aat gat gcg gaa cat tta tcg gat atc gga atc act 450

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Lys	Arg	Leu	Gln	Asn 25	Asp	Ala	Glu	His	Leu 30	Ser	Asp	Ile	Gly	Ile 35	Thr	
gcc Ala	gtc Val	tgg Trp	att Ile 40	cct Pro	ccc Pro	gca Ala	tac Tyr	aaa Lys 45	gga Gly	ttg Leu	agc Ser	caa Gln	tcc Ser 50	gat Asp	aac Asn	498
gga Gly	tac Tyr	gga Gly 55	cct Pro	tat Tyr	gat Asp	ttg Leu	tat Tyr 60	gat Asp	tta Leu	gga Gly	gaa Glu	ttc Phe 65	cag Gln	caa Gln	aaa Lys	546
ggg Gly	acg Thr 70	gtc Val	aga Arg	acg Thr	aaa Lys	tac Tyr 75	ggc Gly	aca Thr	aaa Lys	tca Ser	gag Glu 80	ctt Leu	caa Gln	gat Asp	gcg Ala	594
atc Ile 85	ggc Gly	tca Ser	ctg Leu	cat His	tcc Ser 90	cgg Arg	aac Asn	gtc Val	caa Gln	gta Val 95	tac Tyr	gga Gly	gat Asp	gtg Val	gtt Val 100	642
ttg Leu	aat Asn	cat His	aag Lys	gct Ala 105	ggt Gly	gct Ala	gat Asp	gca Ala	aca Thr 110	gaa Glu	gat Asp	gta Val	act Thr	gcc Ala 115	gtc Val	690
gaa Glu	gtc Val	aat Asn	ccg Pro 120	gcc Ala	aat Asn	aga Arg	aat Asn	cag Gln 125	gaa Glu	act Thr	tcg Ser	gag Glu	gaa Glu 130	tat Tyr	caa Gln	738
atc Ile	aaa Lys	gcg Ala 135	tgg Trp	acg Thr	gat Asp	ttt Phe	cgt Arg 140	ttt Phe	ccg Pro	ggc Gly	cgt Arg	gga Gly 145	aac Asn	acg Thr	tac Tyr	786
agt Ser	gat Asp 150	ttt Phe	aaa Lys	tgg Trp	cat His	tgg Trp 155	tat Tyr	cat His	ttc Phe	gac Asp	gga Gly 160	gcg Ala	gac Asp	tgg Trp	gat Asp	834
gaa Glu 165	tcc Ser	cgg Arg	aag Lys	atc Ile	agc Ser 170	cgc Arg	atc Ile	ttt Phe	aag Lys	ttt Phe 175	cgt Arg	ggg Gly	gaa Glu	gga Gly	aaa Lys 180	882
gcg Ala	tgg Trp	gat Asp	tgg Trp	gaa Glu 185	gta Val	tca Ser	agt Ser	gaa Glu	aac Asn 190	ggc Gly	aac Asn	tat Tyr	gac Asp	tat Tyr 195	tta Leu	930
atg Met	tat Tyr	gct Ala	gat Asp 200	gtt Val	gac Asp	tac Tyr	gac Asp	cac His 205	cct Pro	gat Asp	gtc Val	gtg Val	gca Ala 210	gag Glu	aca Thr	978
aaa Lys	aaa Lys	tgg Trp 215	ggt Gly	atc Ile	tgg Trp	tat Tyr	gcg Ala 220	aat Asn	gaa Glu	ctg Leu	tca Ser	tta Leu 225	gac Asp	ggc Gly	ttc Phe	1026
cgt Arg	att Ile 230	gat Asp	gcc Ala	gcc Ala	aaa Lys	cat His 235	att Ile	aaa Lys	ttt Phe	tca Ser	ttt Phe 240	ctg Leu	cgt Arg	gat Asp	tgg Trp	1074
gtt Val 245	cag Gln	gcg Ala	gtc Val	aga Arg	cag Gln 250	gcg Ala	acg Thr	gga Gly	aaa Lys	gaa Glu 255	atg Met	ttt Phe	acg Thr	gtt Val	gcg Ala 260	1122
gag Glu	tat Tyr	tgg Trp	cag Gln	aat Asn 265	aat Asn	gcc Ala	ggg Gly	aaa Lys	ctc Leu 270	gaa Glu	aac Asn	tac Tyr	ttg Leu	aat Asn 275	aaa Lys	1170
aca Thr	agc Ser	ttt Phe	aat Asn 280	caa Gln	tcc Ser	gtg Val	ttt Phe	gat Asp 285	gtt Val	ccg Pro	ctt Leu	cat His	ttc Phe 290	aat Asn	tta Leu	1218
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10182.ST25															
Gln	Ala	Ala	Ser	Ser	Gln	Gly	Gly	Gly	Tyr	Asp	Met	Arg	Arg	Leu	Leu
		295					300					305			
gac	ggt	acc	gtt	gtg	tcc	agg	cat	ccg	gaa	aag	gcg	gtt	aca	ttt	gtt
Asp	Gly	Thr	Val	Val	Ser	Arg	His	Pro	Glu	Lys	Ala	Val	Thr	Phe	Val
	310					315					320				1314
gaa	aat	cat	gac	aca	cag	ccg	gga	cag	tca	ttg	gaa	tcg	aca	gtc	caa
Glu	Asn	His	Asp	Thr	Gln	Pro	Gly	Gln	Ser	Leu	Glu	Ser	Thr	Val	Gln
	325				330					335					340
act	tgg	ttt	aaa	ccg	ctt	gca	tac	gcc	ttt	att	ttg	aca	aga	gaa	tcc
Thr	Trp	Phe	Lys	Pro	Leu	Ala	Tyr	Ala	Phe	Ile	Leu	Thr	Arg	Glu	Ser
				345					350					355	1410
ggt	tat	cct	cag	gtg	ttc	tat	ggg	gat	atg	tac	ggg	aca	aaa	ggg	aca
Gly	Tyr	Pro	Gln	Val	Phe	Tyr	Gly	Asp	Met	Tyr	Gly	Thr	Lys	Gly	Thr
			360					365					370		1458
tcg	cca	aag	gaa	att	ccc	tca	ctg	aaa	gat	aat	ata	gag	ccg	att	tta
Ser	Pro	Lys	Glu	Ile	Pro	Ser	Leu	Lys	Asp	Asn	Ile	Glu	Pro	Ile	Leu
		375					380					385			1506
aaa	gcg	cgt	aag	gag	tac	gca	tac	ggg	ccc	cag	cac	gat	tat	att	gac
Lys	Ala	Arg	Lys	Glu	Tyr	Ala	Tyr	Gly	Pro	Gln	His	Asp	Tyr	Ile	Asp
	390					395					400				1554
cac	ccg	gat	gtg	atc	gga	tgg	acg	agg	gaa	ggt	gac	agc	tcc	gcc	gcc
His	Pro	Asp	Val	Ile	Gly	Trp	Thr	Arg	Glu	Gly	Asp	Ser	Ser	Ala	Ala
	405				410					415					420
aaa	tca	ggt	ttg	gcc	gct	tta	atc	acg	gac	gga	ccc	ggc	gga	tca	aag
Lys	Ser	Gly	Leu	Ala	Ala	Leu	Ile	Thr	Asp	Gly	Pro	Gly	Gly	Ser	Lys
				425					430				435		1650
cgg	atg	tat	gcc	ggc	ctg	aaa	aat	gcc	ggc	gag	aca	tgg	tat	gac	ata
Arg	Met	Tyr	Ala	Gly	Leu	Lys	Asn	Ala	Gly	Glu	Thr	Trp	Tyr	Asp	Ile
			440					445					450		1698
acg	ggc	aac	cgt	tca	gat	act	gta	aaa	atc	gga	tct	gac	ggc	tgg	gga
Thr	Gly	Asn	Arg	Ser	Asp	Thr	Val	Lys	Ile	Gly	Ser	Asp	Gly	Trp	Gly
		455					460					465			1746
gag	ttt	cat	gta	aac	gat	ggg	tcc	gtc	tcc	att	tat	gtt	cag	aaa	taa
Glu	Phe	His	Val	Asn	Asp	Gly	Ser	Val	Ser	Ile	Tyr	Val	Gln	Lys	
	470					475					480				1794
ggtaataaaaa aaacacctcc aagctgagtg cgggtatcag cttggagggtg cgtttatttt															1854
ttcagccgta tgacaaggtc ggcatcaggt gtgacaaata cggtatgctg gctgtcatag															1914
gtgacaaatc cgggtttttgc gccgtttggc tttttcacat gtctgatttt tgtataatca															1974
acaggcacgg agccggaatc tttcgccttg gaaaaataag cggcgatcgt agctgcttcc															2034
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<210> 10

<211> 483

<212> PRT

<213> Bacillus amyloliquefaciens

10182.ST25

<400> 10

Val Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Thr Pro Asn Asp
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Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ala Glu His Leu Ser Asp
 20 25 30

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

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

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

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

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

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

Phe Thr Val Ala Glu Tyr Trp Gln Asn Asn Ala Gly Lys Leu Glu Asn
 260 265 270

10182.ST25

Tyr Leu Asn Lys Thr Ser Phe Asn Gln Ser Val Phe Asp Val Pro Leu
 275 280 285
 His Phe Asn Leu Gln Ala Ala Ser Ser Gln Gly Gly Gly Tyr Asp Met
 290 295 300
 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
 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
 Thr Lys Gly Thr Ser Pro Lys Glu Ile Pro Ser Leu Lys Asp Asn Ile
 370 375 380
 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
 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
 Asp Gly Trp Gly Glu Phe His Val Asn Asp Gly Ser Val Ser Ile Tyr
 465 470 475 480
 Val Gln Lys

<210> 11

<211> 1458

<212> DNA

<213> Bacillus sp.

10182.ST25

<220>

<221> CDS

<222> (1)..(1458)

<223> AA560

<400> 11

cac	cat	aat	ggt	acg	aac	ggc	aca	atg	atg	cag	tac	ttt	gaa	tgg	tat	48
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1				5				10					15			

cta	cca	aat	gac	gga	aac	cat	tgg	aat	aga	tta	agg	tct	gat	gca	agt	96
Leu	Pro	Asn	Asp	Gly	Asn	His	Trp	Asn	Arg	Leu	Arg	Ser	Asp	Ala	Ser	
			20					25					30			

aac	cta	aaa	gat	aaa	ggg	atc	tca	gcg	gtt	tgg	att	cct	cct	gca	tgg	144
Asn	Leu	Lys	Asp	Lys	Gly	Ile	Ser	Ala	Val	Trp	Ile	Pro	Pro	Ala	Trp	
		35					40					45				

aag	ggt	gcc	tct	caa	aat	gat	gtg	ggg	tat	ggt	gct	tat	gat	ctg	tat	192
Lys	Gly	Ala	Ser	Gln	Asn	Asp	Val	Gly	Tyr	Gly	Ala	Tyr	Asp	Leu	Tyr	
	50					55					60					

gat	tta	gga	gaa	ttc	aat	caa	aaa	gga	acc	att	cgt	aca	aaa	tat	gga	240
Asp	Leu	Gly	Glu	Phe	Asn	Gln	Lys	Gly	Thr	Ile	Arg	Thr	Lys	Tyr	Gly	
65					70					75					80	

acg	cgc	aat	cag	tta	caa	gct	gca	gtt	aac	gcc	ttg	aaa	agt	aat	gga	288
Thr	Arg	Asn	Gln	Leu	Gln	Ala	Ala	Val	Asn	Ala	Leu	Lys	Ser	Asn	Gly	
				85					90					95		

att	caa	gtg	tat	ggc	gat	gtt	gta	atg	aat	cat	aaa	ggg	gga	gca	gac	336
Ile	Gln	Val	Tyr	Gly	Asp	Val	Val	Met	Asn	His	Lys	Gly	Gly	Ala	Asp	
			100					105					110			

gct	acc	gaa	atg	gtt	agg	gca	gtt	gaa	gta	aac	ccg	aat	aat	aga	aat	384
Ala	Thr	Glu	Met	Val	Arg	Ala	Val	Glu	Val	Asn	Pro	Asn	Asn	Arg	Asn	
		115					120					125				

caa	gaa	gtg	tcc	ggt	gaa	tat	aca	att	gag	gct	tgg	aca	aag	ttt	gac	432
Gln	Glu	Val	Ser	Gly	Glu	Tyr	Thr	Ile	Glu	Ala	Trp	Thr	Lys	Phe	Asp	
	130					135					140					

ttt	cca	gga	cga	ggt	aat	act	cat	tca	aac	ttc	aaa	tgg	aga	tgg	tat	480
Phe	Pro	Gly	Arg	Gly	Asn	Thr	His	Ser	Asn	Phe	Lys	Trp	Arg	Trp	Tyr	
145					150					155					160	

cac	ttt	gat	gga	gta	gat	tgg	gat	cag	tca	cgt	aag	ctg	aac	aat	cga	528
His	Phe	Asp	Gly	Val	Asp	Trp	Asp	Gln	Ser	Arg	Lys	Leu	Asn	Asn	Arg	
				165					170					175		

att	tat	aaa	ttt	aga	ggt	gat	gga	aaa	ggg	tgg	gat	tgg	gaa	gtc	gat	576
Ile	Tyr	Lys	Phe	Arg	Gly	Asp	Gly	Lys	Gly	Trp	Asp	Trp	Glu	Val	Asp	
			180					185					190			

aca	gaa	aac	ggt	aac	tat	gat	tac	cta	atg	tat	gca	gat	att	gac	atg	624
Thr	Glu	Asn	Gly	Asn	Tyr	Asp	Tyr	Leu	Met	Tyr	Ala	Asp	Ile	Asp	Met	
		195					200					205				

gat	cac	cca	gag	gta	gtg	aat	gag	cta	aga	aat	tgg	ggt	gtt	tgg	tat	672
Asp	His	Pro	Glu	Val	Val	Asn	Glu	Leu	Arg	Asn	Trp	Gly	Val	Trp	Tyr	
	210					215					220					

acg	aat	aca	tta	ggc	ctt	gat	ggt	ttt	aga	ata	gat	gca	gta	aaa	cat	720
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10182.ST25															
Thr 225	Asn	Thr	Leu	Gly	Leu 230	Asp	Gly	Phe	Arg	Ile 235	Asp	Ala	Val	Lys	His 240
ata Ile	aaa Lys	tac Tyr	agc Ser	ttt Phe 245	act Thr	cgt Arg	gat Asp	tgg Trp	att Ile 250	aat Asn	cat His	gtt Val	aga Arg	agt Ser 255	gca Ala
act Thr	ggc Gly	aaa Lys	aat Asn 260	atg Met	ttt Phe	gcg Ala	gtt Val	gcg Ala 265	gaa Glu	ttt Phe	tgg Trp	aaa Lys	aat Asn 270	gat Asp	tta Leu
ggg Gly	gct Ala	att Ile 275	gaa Glu	aac Asn	tat Tyr	tta Leu	aac Asn 280	aaa Lys	aca Thr	aac Asn	tgg Trp	aac Asn 285	cat His	tca Ser	gtc Val
ttt Phe	gat Asp 290	gtt Val	ccg Pro	ctg Leu	cac His	tat Tyr 295	aac Asn	ctc Leu	tat Tyr	aat Asn	gct Ala 300	tca Ser	aaa Lys	agc Ser	gga Gly
ggg Gly 305	aat Asn	tat Tyr	gat Asp	atg Met	agg Arg 310	caa Gln	ata Ile	ttt Phe	aat Asn	ggg Gly 315	aca Thr	gtc Val	gtg Val	caa Gln	aga Arg 320
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gaa Glu	gaa Glu	gct Ala	tta Leu 340	gag Glu	tct Ser	ttt Phe	gtt Val	gaa Glu 345	gaa Glu	tgg Trp	ttc Phe	aaa Lys	cca Pro 350	tta Leu	gcg Ala
tat Tyr	gct Ala	ttg Leu 355	aca Thr	tta Leu	aca Thr	cgt Arg	gaa Glu 360	caa Gln	ggc Gly	tac Tyr	cct Pro	tct Ser 365	gta Val	ttt Phe	tat Tyr
gga Gly 370	gat Asp	tat Tyr	tat Tyr	ggc Gly	att Ile	cca Pro 375	acg Thr	cat His	ggg Gly	gta Val	cca Pro 380	gcg Ala	atg Met	aaa Lys	tcg Ser
aaa Lys 385	att Ile	gac Asp	ccg Pro	att Ile	cta Leu 390	gaa Glu	gcg Ala	cgt Arg	caa Gln	aag Lys 395	tat Tyr	gca Ala	tat Tyr	gga Gly	aga Arg 400
caa Gln	aat Asn	gac Asp	tac Tyr	tta Leu 405	gac Asp	cat His	cat His	aat Asn	atc Ile 410	atc Ile	ggg Gly	tgg Trp	aca Thr	cgt Arg 415	gaa Glu
ggg Gly	aat Asn	aca Thr	gca Ala 420	cac His	ccc Pro	aac Asn	tcc Ser	ggg Gly 425	tta Leu	gct Ala	act Thr	atc Ile	atg Met 430	tcc Ser	gat Asp
ggg Gly	gca Ala	gga Gly 435	gga Gly	aat Asn	aag Lys	tgg Trp	atg Met 440	ttt Phe	gtt Val	ggg Gly	cgt Arg	aat Asn 445	aaa Lys	gct Ala	ggg Gly
caa Gln	gtt Val 450	tgg Trp	acc Thr	gat Asp	atc Ile	act Thr 455	gga Gly	aat Asn	cgt Arg	gca Ala	ggg Gly 460	act Thr	gtt Val	acg Thr	att Ile
aat Asn 465	gct Ala	gat Asp	gga Gly	tgg Trp	ggg Gly 470	aat Asn	ttt Phe	tct Ser	gta Val	aat Asn 475	gga Gly	gga Gly	tca Ser	gtt Val	tct Ser 480
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10182.ST25

<210> 12

<211> 485

<212> PRT

<213> Bacillus sp.

<400> 12

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
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Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Ser Asp Ala Ser
 20 25 30

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

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 Ile Arg Thr Lys Tyr Gly
 65 70 75 80

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

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

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

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

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

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

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

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met
 195 200 205

Asp His Pro Glu Val Val Asn Glu Leu Arg Asn Trp Gly Val Trp Tyr
 210 215 220

10182.ST25

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

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
 260 265 270

Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val
 275 280 285

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 Gln Ile Phe Asn Gly Thr Val Val Gln Arg
 305 310 315 320

His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
 325 330 335

Glu Glu Ala Leu Glu Ser Phe Val Glu Glu Trp Phe Lys Pro Leu Ala
 340 345 350

Tyr Ala Leu Thr Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
 355 360 365

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

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

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

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

Gly Ala Gly Gly Asn Lys Trp Met Phe Val Gly Arg Asn Lys Ala Gly
 435 440 445

Gln Val Trp Thr Asp Ile Thr Gly Asn Arg Ala Gly Thr Val Thr Ile
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Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
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Ile Trp Val Asn Lys
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10182.ST25

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<400> 13

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
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Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Asn Ser Asp Ala Ser
 20 25 30

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

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

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
 100 105 110

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

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

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
 180 185 190

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met
 195 200 205

Asp His Pro Glu Val Val Asn Glu Leu Arg Asn Trp Gly Val Trp Tyr
 210 215 220

10182.ST25

Thr Asn Thr Leu Gly Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
 225 230 235 240
 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
 260 265 270
 Gly Ala Ile Glu Asn Tyr Leu Gln Lys Thr Asn Trp Asn His Ser Val
 275 280 285
 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
 His Pro Ser His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
 325 330 335
 Glu Glu Ala Leu Glu Ser Phe Val Glu Glu Trp Phe Lys Pro Leu Ala
 340 345 350
 Tyr Ala Leu Thr Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
 355 360 365
 Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Arg Ser
 370 375 380
 Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Lys Tyr Ala Tyr Gly Lys
 385 390 395 400
 Gln Asn Asp Tyr Leu Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
 405 410 415
 Gly Asn Thr Ala His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
 420 425 430
 Gly Ala Gly Gly Ser Lys Trp Met Phe Val Gly Arg Asn Lys Ala Gly
 435 440 445
 Gln Val Trp Ser Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
 450 455 460
 Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
 465 470 475 480
 Ile Trp Val Asn Lys
 485