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

(57) **Abrégé/Abstract:**

The present invention relates to variants (mutants) of polypeptides, in particular Termamyl-like alpha-amylases, 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, Ca²⁺ dependency, specific activity, in particular laundry and dish-wash applications.



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

(57) Abstract: The present invention relates to variants (mutants) of polypeptides, in particular Termamyl-like alpha-amylases, 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, Ca²⁺ dependency, specific activity, in particular laundry and dish-wash applications.



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Alpha-amylase variants with altered properties**FIELD OF THE INVENTION**

The present invention relates to variants (mutants) of parent Termamyl-like alpha-amylases, 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 pI and improved wash performance, specific activity, stability under, e.g., high temperature and/or low/high pH conditions, in particular at low calcium concentrations, and stability in the presence of detergent, e.g. storage stability in the detergents. 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.

The object of the invention is to provide an improved alpha-amylase, in particular suitable for detergent use.

SUMMARY OF THE INVENTION

The object of the present invention is to provide Termamyl-like amylases which variants 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 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).

5 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:

*36Asp or *36D

for insertion of an aspartic acid in position 36.

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

10 Ala30Asn + Glu34Ser or A30N+E34S

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

15 A30N,E or
A30N or A30E

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
20 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:
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,
25 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.

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 of N or V is present in the wildtype.

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

Characteristics of amino acid residues

Charged amino acids:

35 Asp, Glu, Arg, Lys, His

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

Asp, Glu

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

5 Arg, Lys, His

Neutral amino acids:

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

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

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

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

Thr, Ser, Cys, Gln, Asn

15

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an alignment of the amino acid sequences of thirteen parent Termamyl-like alpha-amylases.

20 DETAILED DESCRIPTION OF THE INVENTION

The object of the present invention is to provide polypeptides, such as enzymes, in particular alpha-amylases, with an alteration in at least one of the following properties relative to said parent polypeptide: substrate specificity, substrate binding, substrate cleavage pattern, thermal stability, pH/activity profile, stability towards oxidation, Ca^{2+} dependency, and specific
25 activity, in particular in laundry and dish-wash applications. The properties will be defined further below.

Polypeptide

Polypeptides according to the invention include proteins with biological activity, antimicrobial
30 activity, and enzymatic activity.

Contemplated enzyme activities include proteases, amylases, CGTases, mannanases, maltogenic amylases, glucoamylases, carbohydrases, transferases, lyases, oxidoreductases, lipases.

In one preferred embodiment the enzyme is an alpha-amylase, in particular a *Bacillus* or
35 *Aspergillus* alpha-amylase. In a preferred embodiment the *Bacillus* alpha-amylase is a Termamyl-like amylases.

Polypeptides with biological activity include, EPO, TPO, growth hormones, regulatory

peptides, blood coagulation factors, antibodies etc.

The Tertiary Structure of SP722 and Modelling the Tertiary Structures of another Termamyl-like alpha-amylase.

5 Mutants of alpha-amylases of the present invention have been found based on the tertiary structure of SP722 shown in APPENDIX 1 of WO 01/66712. Mutants of other polypeptides may be found based on other tertiary structures.

A model of another alkaline Termamyl-like amylase, AA560 has been build based on the SP722 tertiary structure disclosed in APPENDIX 1 of WO 01/66712. The AA560 alpha-amylase
10 is about 87% identical to the template amylase (SP722) and the alignment contains no insertion or deletions.

The findings of the present invention may be applied on Termamyl-like amylases being at least 60% identical, preferably at least 70% identical, more preferably 80% identical, even more preferably 85% identical, even more preferably 90% identical, even more 95% identical, even
15 more 97% identical, even more 99% identical to the Termamyl-like alpha-amylase shown in SEQ ID NO: 12. In a preferably the findings may be used on alkaline Termamyl-like alpha-amylases, especially alkaline alpha-amylases of the same length, without additional amino residues or gaps in an aligned primary structure in comparison to SP722 (SEQ ID NO: 4 shown as number 7 in the alignment in Fig. 1). Especially, the finding may be used on the following alkaline Termamyl-
20 like alpha-amylases: SP690 (SEQ ID NO: 2), SP722 (SEQ ID NO: 4), AA560 (SEQ ID NO: 12), #707 alpha-amylase (SEQ ID NO: 13), the KSM AP 1378 alpha-amylase is disclosed in WO 97/00324, the #SP7-7 alpha-amylase is disclosed in WO 02/10356, or fragment or truncated forms thereof. The latter mentioned alkaline alpha-amylases have very similar tertiary crystal structure around the above-mentioned interactions zones, and have the same primary structure
25 length 485 amino acids.

Contrary hereto, for instance, Termamyl (shown as sequence number 1 in the alignment in Fig. 1) lacks two amino acid residues (positions 1 and 2); has gaps in positions 174 and 181-182; and has three additional amino acid residues in positions 378-381 when aligned with SP722.

30 BAN (shown as sequence number 4 in the alignment in Fig. 1) lacks five amino acid residues (positions 1-4 and 488); has gaps in positions 174 and 181-182; and has three additional amino acid residues in positions 378-381 if aligned with SP722.

BSG (shown as sequence number 3 in the alignment in Fig. 1) lacks one amino acid residues (position 1); and has 31 additional amino acid residues in positions 489-519 if aligned with
35 SP722.

KSM-K36 and KSM-K38 (EP 1,022,334-A) lack five amino acid residues (positions 1 and 2) and has gaps in positions 174 and 181-182 when aligned with SP722.

AA180, AA20 and Amrk385 (Danish patent application no. PA 2000 00347 or PCT/DK01/00133) have one additional amino acid in position 261 when aligned with SP722.

Below it is described how to model a Termamyl-like alpha-amylase from another alpha-amylase. This method can be extrapolated to other polypeptides as for instance the above-mentioned.

Modelling of Termamyl-like Alpha-amylases

WO 96/23874 provides the tertiary structure (3D Structure), X-ray crystal structural data for a Termamyl-like alpha-amylase, which consists of the 300 N-terminal amino acid residues of the *B. amyloliquefaciens* alpha-amylase (BANTM) and amino acids 301-483 of the C-terminal end of the *B. licheniformis* alpha-amylase (SEQ ID NO: 8). WO 96/23874 further describes methodology for designing (modelling), on the basis of an analysis of the structure of a parent Termamyl-like alpha-amylase, variants of the parent Termamyl-like alpha-amylase which exhibit altered properties relative to the parent.

Other Termamyl-like structures may be modelled in accordance with WO 96/23874, which is hereby incorporated by reference.

In connection with obtaining variant of the present invention the AA560 tertiary structure was designed (modelled) based on the tertiary structure of SP722 (disclosed in APPENDIX 1) as described in Example 1. The structure of other Termamyl-like alpha-amylases (e.g., those disclosed herein) may be built analogously.

Termamyl-like alpha-amylases

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

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

Table 1

	BLA	BAN	AMY1048	BSG	AA560	sp.707	sp.7-7	AMRK385	SP690	VI-AP1378	SP722	AAI10	K38
BLA	100,0	80,8	65,2	65,4	68,1	67,9	67,1	70,6	68,7	68,9	70,2	70,2	62,5
BAN	80,6	100,0	64,9	65,1	66,3	66,3	65,7	67,6	66,5	66,5	68,0	68,2	59,5
AMY1048	53,8	53,6	100,0	75,6	54,6	54,6	55,0	57,0	56,1	55,8	55,6	56,5	48,1
BSG	61,4	61,2	86,0	100,0	62,3	62,5	63,1	64,1	63,7	63,7	63,1	65,6	55,2
AA560	67,8	66,2	66,0	66,2	100,0	95,5	94,6	89,7	87,0	86,0	86,8	78,6	63,7
sp.707	67,6	66,2	66,0	66,4	95,5	100,0	92,8	90,5	87,6	86,4	86,2	79,8	63,5
Sp.7-7	66,8	65,6	66,4	67,0	94,6	92,8	100,0	89,1	88,5	87,2	87,0	79,2	63,7
AMRK385	70,2	67,3	68,7	67,9	89,5	90,3	88,9	100,0	89,3	88,9	87,5	81,9	65,4
SP690	68,5	66,4	67,8	67,6	87,0	87,6	88,5	89,5	100,0	95,1	87,2	81,2	65,6
KSM-AP1378	68,7	66,4	67,4	67,6	86,0	86,4	87,2	89,1	95,1	100,0	86,6	80,6	66,2
SP722	69,9	67,8	67,2	67,0	86,8	86,2	87,0	87,6	87,2	86,6	100,0	80,6	66,0
AAI10	69,9	68,0	68,3	69,7	78,6	79,8	79,2	82,1	81,2	80,6	80,6	100,0	68,5
K38	62,9	60,0	58,8	59,2	64,4	64,2	64,4	66,3	66,3	66,9	66,7	69,2	100,0

For instance, the *B. licheniformis* alpha-amylase comprising the amino acid sequence shown in SEQ ID NO: 8 (commercially available as TermamylTM) 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 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
5 AA560 alpha-amylase derived from *Bacillus* sp. and shown in SEQ ID NO: 12, and the #707 alpha-amylase derived from *Bacillus* sp. 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). Also the K38 and K38 alpha-amylases disclosed in EP 1,022,334 are contemplated according
10 to the invention.

Other alpha-amylases are shown in SEQ ID NOS: 13, 14, 15, 16, 17, and 18.

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
15 comprised in the products sold under the following tradenames: Optitherm™ and Takatherm™ (available from Solvay); Maxamyl™ (available from Gist-brocades/Genencor), Spezym AA™ and Spezyme Delta AA™ (available from Genencor), and Keistase™ (available from Daiwa), Purastar™ ST 5000E, PURASTRA™ HPAM L (from Genencor Int.).

Because of the substantial homology found between these alpha-amylases, they are
20 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, 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
25 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, 13, 14, 15, 16, 17, and 18 herein are considered to be "Termamyl-like alpha-amylase". Other Termamyl-like alpha-amylases are alpha-amylases i)
30 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 97%, at least 99% homology with at least one of said amino acid sequences shown in SEQ ID NOS: 2, 4, 6, 8, 10, 12, 13, 14, 15, 16, 17 and 18 and/or ii) 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, 11 and of the present specification (which encoding sequences encode the amino acid sequences shown
35 in SEQ ID NOS: 2, 4, 6, 8, 10 and 12 herein, respectively)

Also Termamyl amylases consisting of 1) a catalytic domain with high homology to Termamyl and 2) of a carbohydrate binding domain (CBM) should be understood as included

in this application. The Binding domain may be located in either N-terminal relative to the sequence of the catalytic domain or C-terminal relative to the catalytic domain, there might be more than one CBM located either N-or C-terminal or both. The amylases with CBM might come from natural sources or may be the results of genetic engineering fusing the gene coding an amylase with a gene coding a CBM.

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 polypeptide, such as the Termamyl-like alpha-amylase in accordance with property ii) 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 prehybridizing 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 100mM 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 all Termamyl-like alpha-amylases, 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: 10.

In a preferred embodiment the parent Termamyl-like alpha amylase is SP722 or BSG or AA560 including any of SP722+R181*+G182*, SP722+D183*+G184*; SP722+D183*+G184*+N195F; SP722+D183*+G184*+M202L; SP722+D183*+G184*+N195F+M202L; SP722+D183*+G184*+R181Q; SP722+D183*+G184*+R118K+N195F+R320K+R458K; 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; AA560+D183*+G184*+R118K+N195F+R320K+R458K.

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

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 one alpha-amylase.

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 those 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*. For instance, the parent α -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* α -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).

Yet another suitable parent hybrid alpha-amylase consist of the sequence of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 13, 14, 15, 16, 17, or 18, and the last 99 amino acids of SEQ ID NO:13 (AMY1048)

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 33 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
 5 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;
 10 H156Y+A181T+A209V+Q264S; H156Y+A181T+Q264S; H156Y+Q264S;
 H156Y+A181T+N190F+Q264S; H156Y+A181T+N190F; H156Y+A181T+N190F+A209V. These hybrids are also considered to be part of the invention.

In a preferred embodiment the parent Termamyl-like alpha amylase is LE174 including any of
 LE174+G48A+T49I+G107A+I201F; LE174+M197L; or
 15 LE174+G48A+T49I+G107A+M197L+I201F.

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., LE174, with G48A+T49I+G107A+I201F.

Construction of variants of the invention

The construction of the variant of interest may be accomplished by cultivating a microorganism comprising a DNA sequence encoding the variant under conditions which are conducive for producing the variant. The variant may then subsequently be recovered from the
 25 resulting culture broth. This is described in detail further below.

Altered properties

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

As mentioned above the invention relates to Termamyl-like alpha-amylases with altered properties, in particular at high temperatures and/or at low pH, in particular at low calcium concentrations.

In the context of the present invention "high temperature" means temperatures from 70-
 35 120°C, preferably 80-100°C, especially 85-95°C.

In the context of the present invention the term "low pH" means from a pH in the range from 4-6, preferably 4.2-5.5, especially 4.5-5.

In the context of the present invention the term "high pH" means from a pH in the range from 8-11, especially 8.5-10.6.

In the context of the present invention the term "low calcium concentration" means free calcium levels lower than 60 ppm, preferably 40 ppm, more preferably 25 ppm, especially 5 ppm calcium.

Parent Termamyl-like alpha-amylase specifically contemplated in connection with going through the specifically contemplated altered properties are the above mentioned parent Termamyl-like alpha-amylase and parent hydrid Termamyl-like alpha-amylases. The SP722 alpha-amylase is used as the starting point, but corresponding positions in, e.g., the Termamyl, BSG, BAN, AA560, SP690, AA180, KSM AP1378, SP7-7 and #707, K38, and K36 should be understood as disclosed too.

Design of improved oxidation stable amylase variants:

M197 in SEQ ID NO: 8 or the equivalent M202 in SEQ ID NO: 12 has been shown to increase the stability in the presence of bleaching agents like e.g. perborate etc. in detergents. Also mutation of M15 in SEQ ID NO: 8 has shown some effect but for SEQ ID NO: 2, 4, 6, 10, and 12 and other amylases which do not have a corresponding methionine at position equivalent to M15, other residues, in particular other Methionines, have been found to increase the stability beyond what is observed for M202. These include but are not limited to M9, M10, M105, M116 (not present in SP690, SP722, AMRK385) M202, M208, M261, M309, M323 (only in AA560, SP722), M382, M410 (SP.7-7), M430, M440, in SEQ ID NO: 12, 17, and 18, whereas in SEQ ID NO: 16 (AAI-10) the most interesting positions are: M10, M105, M202, M208, M246, M286, M309, M430, M440, M454 and whereas in SEQ ID NO: 14(Amrk385) the most interesting positions are: M9, M10, M105, M202, M208, M262, M310, M383, M431, M441, and whereas in SEQ ID NO: 15 (K38) the most interesting positions are: M7, M8, M103, M107, M277, M281, M304, M318, M380, M425, M435. The most preferred substitutions are: M9L,I, M10L, M105L,I,F, M116N,D,L,I,F,W,R,K, M202L,I,T,V, M208F,Y, L,I, M261L,I, M309L,I, M323L,I,S,T,A,Q,E,N,D, M382L,I,Y,F,K, M410L,I,V, M430L,I, M440L,I,F,Y.

As stated above M202 has been shown to be important for the stability in the presence of bleaching agents. However mutating M202 to substitutions preferred for stability, reduces the activity of the amylase. To re-activate the amylase, substitutions along the putative substrate binding cleft has shown to be beneficial for the activity. These include among others: T193, K269, N270, L272, Y295, N296, N299, S303, Y304, Q311, N314, G315, Q319, and A339. The preferred mutations being: T193S,N,D,E,Q, K269S,Q, N270F,Y,D, L272I,V,A, Y295F,N,D,Q,E, N296K,Q,E, N299F,Y,Q,T, S303Q,K, Y304F,R,K, Q311N,Q,K,R,T,S,Y,F, N314D,S,T,Q, G315N,S,T, Q319E,K,S,T, A339S,T.

The optimal enzyme for washing application has to fulfill several criteria to work

optimally. It should be stable in the detergent matrix prior to usage, it should be stable during wash and it should be highly active during wash. There are several examples reported for optimizing each of these criteria but as oxidation stabile amylases are less active and activated amylases are less stable, it is the scope of this invention to identify the optimal combination of substitutions fulfilling all three demands. The preferred combinations are:

M9L+M202I

M9L+M202I+M323T

M9L+M202I+M323T+M382Y

M202I+Y295F

M202I+A339S

M9L+M202I+Y295F+A339S

M9L+M202I+Y295F

M9L+M202I+A339S

M9L+M202I+Y295F+A339S

M9L+M202I+Y295F+A339S+E345R

M9L+G149A+M202I+Y295F+A339S+E345R

M9L+M202L

M9L+M202L+M323T

M9L+M202L+M323T+M382Y

M202L+Y295F

M202L+A339S

M9L+M202L+Y295F+A339S

M9L+M202L+Y295F

M9L+M202L+A339S

M9L+M202L+Y295F+A339S

M9L+M202L+Y295F+A339S+E345R

M9L+G149A+M202L+Y295F+A339S+E345R

M9L+M202T

M9L+M202T+M323T

M9L+M202T+M323T+M382Y

M202T+Y295F

M202T+A339S

M9L+M202T+Y295F+A339S

M9L+M202T+Y295F

M9L+M202T+A339S

M9L+M202T+Y295F+A339S

M9L+M202T+Y295F+A339S+E345R

M9L+G149A+M202T+Y295F+A339S+E345R

M9L+G149A+M202I+V214T+Y295F+N299Y+M323T+A339S+E345R

M9L+G149A+M202L+V214I+Y295F+M323T+A339S+E345R+M382Y

M9L+G149A+G182T+G186A+M202I+V214I+Y295F+N299Y+M323T+A339S

5 M9L+G149A+G182T+G186A+M202L+T257I+Y295F+N299Y+M323T+A339S+E345R

M9L+G149A+M202L+V214T+Y295F+N299Y+M323T+A339S+E345R

M9L+G149A+M202I+V214I+Y295F+M323T+A339S+E345R+M382Y

M9L+G149A+G182T+G186A+M202L+V214I+Y295F+N299Y+M323T+A339S

M9L+G149A+G182T+G186A+M202I+T257I+Y295F+N299Y+M323T+A339S+E345R

10 M9L+G149A+M202I+V214T+Y295F+N299Y+M323T+A339S+E345R+N471E

M9L+G149A+M202L+V214I+Y295F+M323T+A339S+E345R+M382Y+N471E

M9L+G149A+G182T+G186A+M202I+V214I+Y295F+N299Y+M323T+A339S+N471E

M9L+G149A+G182T+G186A+M202L+T257I+Y295F+N299Y+M323T+A339S+E345R+N471E

15 In the first aspect a variant of a parent Termamyl-like alpha-amylase, comprising an alteration at one or more positions selected from the group of:

26, 30, 33, 82, 37, 106, 118, 128, 133, 149, 150, 160, 178, 182, 186, 193, 203, 214, 231, 256,

257, 258, 269, 270, 272, 283, 295, 296, 298, 299, 303, 304, 305, 311, 314, 315, 318, 319,

339, 345, 361, 378, 383, 419, 421, 437, 441, 444, 445, 446, 447, 450, 461, 471, 482, 484,

wherein

20 (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,

25 (b) the variant has alpha-amylase activity, and

(c) each position corresponds to a position of the amino acid sequence of the parent alpha-amylase having the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid sequence of AA560 shown in SEQ ID NO: 12.

30 In a preferred embodiment the variant of the invention (using SEQ ID NO: 12 for the numbering) has one or more of the following mutations/substitutions:

R26S, D30N, N33D, R82H, K37T, N106D, K118Q, N128Y, G133E,A, G149A,N, N150H,Q, Y160F, Y178F, G182T, G186A, T193S,N,D,E,Q, Y203L, V214I,T, D231N, G256K, T257I, G258D, K269S,Q, N270F,Y,D, L272I,V,A, N283D, Y295F,N,D,Q,E, N296K,Q,E, Y298F,H, N299F,Y,Q,T, S303Q,K, Y304F,R,K, G305D, Q311N,Q,K,R,T,S,Y,F, N314D,S,T,Q, 35 G315N,S,T, V318L, Q319E,K,S,T, A339S,T, E345N,R, Q361E, G378K, K383R, T419N, H421Y, N437H, F441L, R444E,Y, N445Q, K446R, A447Y, V450T, T461P, N471E, W482Y, N484Q.

Preferred double, triple and multi-mutations – using SEQ ID NO: 12 as the basis for the numbering - include:

M9L+M202I,

M9L+M202I+M323T,

5 M9L+M202I+323T+M382Y,

M9L+M202I+Y295F+A339S,

M9L+M202I+Y295F,

M9L+M202I+A339S,

M9L+M202I+Y295F+A339S,

10 M9L+M202I+Y295F+A339S+E345R,

M9L+G149A+M202I+Y295F+A339S+E345R,

M9L+M202L,

M9L+M202L+M323T,

M9L+M202L+M232T+M382Y,

15 M9L+M202L+Y295F+A339S,

M9L+M202L+Y295F,

M9L+M202L+A339S,

M9L+M202L+Y295F+A339S,

M9L+M202L+Y295F+A339S, E345R,

20 M9L+G149A+M202L+Y295F+A339S+E345R,

M9L+M202T,

M9L+M202T+M323T,

M9L+M202T+M323T+M382Y,

M9L+M202T+Y295F+A339S,

25 M9L+M202T+Y295F,

M9L+M202T+A339S,

M9L+M202T+Y295F+A339S,

M9L+M202T+Y295F+A339S+E345R,

M9L+G149A+M202T+Y295F+A339S+E345R,

30 M9L+G149A+M202I+V214T+Y295F+N299Y+M323T+A339S+E345R,

M9L+G149A+M202L+V214I+Y295F+M323T+A339S+E345R+M382Y,

M9L+G149A+G182T+G186A+M202I+V214I+Y295F+N299Y+M323T+A339S,

M9L+G149A+G182T+G186A+M202L+T257I+Y295F+N299Y+M323T+A339S+E345R,

M9L+G149A+M202L+V214T+Y295F+N299Y+M323T+A339S+E345R,

35 M9L+G149A+M202I+V214I+Y295F+M323T+A339S+E345R+M382Y,

M9L+G149A+G182T+G186A+M202L+V214I+Y295F+N299Y+M323T+A339S,

M9L+G149A+G182T+G186A+M202I+T257I+Y295F+N299Y+M323T+A339S+E345R,

M9L+G149A+M202I+V214T+Y295F+N299Y+M323T+A339S+E345R+N471E,
M9L+G149A+M202L+V214I+Y295F+M323T+A339S+E345R+M382Y+N471E,
M9L+G149A+G182T+G186A+M202I+V214I+Y295F+N299Y+M323T+A339S+N471E,
M9L+G149A+G182T+G186A+M202L+T257I+Y295F+N299Y+M323T+A339S+E345R+N471E,
5 M202L+M105F+M208F,
G133E+M202L+Q361E,
G133E+M202L+R444E,
M202L+Y295F,
M202L+A339S,
10 M202L+M323T,
M202L+M323T+M309L,
M202L+M323T+M430I,
M202L+V214T+R444Y,
M202L+N283D+Q361E,
15 M202L+M382Y+K383R,
M202L+K446R+N484Q,
M202I+Y295F,
M202I+A339S,
M202I+M105F+M208F,
20 G133E+M202I+Q361E,
G133E+M202I+R444E,
M202I+M202I+M323T,
M202I+M202I+M323T+M309L,
M202I+M323T+M430I,
25 M202I+V214T+R444Y,
M202I+N283D+Q361E,
M202I+M382Y+K383R,
M202I+K446R+N484Q,
M202V+M105F+M208F,
30 G133E+M202V+Q361E,
G133E+M202V+R444E,
M202V+M323T,
M202V+M323T+M309L,
M202V+M323T+M430I,
35 M202V+M323T+M9L,
M202V+V214T+R444Y,
M202V+N283D+Q361E,

M202V+M382Y+K383R,
 M202V+K446R+N484Q,
 M202T+M105F+M208F,
 G133E+M202T+Q361E,
 5 G133E+M202T+R444E,
 M202T+Y295F,
 M202T+A339S,
 M202T+M323T,
 M202T+M323T+M309L,
 10 M202T+M323T+M430I,
 M202T+M323T+M9L,
 M202T+V214T+R444Y,
 M202T+N283D+Q361E,
 M202T+A339S,
 15 M202T+Y295F
 M202T+N299F,Y,
 M202T+M382Y+K383R,
 M202T+K446R+N484Q

20 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
 25 solution) calcium concentrations below 60 ppm, include any of the mutations listed in the "Altered Properties" section. The stability may be determined as described in the "Materials & Methods" section below.

Ca²⁺ stability

30 Altered Ca²⁺ stability means the stability of the enzyme under Ca²⁺ depletion has been 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 "Altered properties" section.

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, preferably 20-50°C, especially 30-40°C, include any of the mutations listed in the "Altered properties" section. The specific activity may be determined as described in the "Material & Methods" section below.

Oxidation stability

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.

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.

Wash performance

Important positions and mutations with respect to obtaining variants with improved wash performance at especially neutral to high pH, i.e., pH 6-11, preferably 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.

Methods for preparing alpha-amylase variants of the invention

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

Cloning a DNA sequence encoding an alpha-amylase

The DNA sequence encoding a parent alpha-amylase may be isolated from any cell or 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 oligonucleotide probes may be synthesized and used to identify alpha-amylase-encoding clones from a 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 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.

Random Mutagenesis

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

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

In relation to the above, a further aspect of the present invention relates to a method for generating a variant of a parent alpha-amylase, e.g., wherein the variant exhibits a reduced capability of cleaving an oligo-saccharide substrate close to the branching point, and further exhibits improved substrate specificity and/or improved specific activity relative to the parent, the method:

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

Step (a) of the above method of the invention is preferably performed using doped primers. For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents. The mutagenizing agent may, e.g., be one, which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) ir-radiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties. When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions, which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the alpha-amylase enzyme by any published technique, using e.g., PCR, LCR or any DNA polymerase and ligase as deemed appropriate. Preferably, the doping is carried out using "constant random doping", in which the percentage of wild type and mutation in each position is predefined. Furthermore, the doping may be directed toward a preference for the introduction of certain nucleotides, and thereby a preference for the introduction of one or more specific amino acid residues. The doping may be made, e.g., so as to allow for the introduction of 90% wild type and 10% mutations in each position. An additional consideration in the choice of a doping scheme is based on genetic as well as protein-structural constraints. The doping scheme may be made by using the DOPE program, which, inter alia, ensures that introduction of stop codons is avoided. When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent alpha-amylase is subjected to PCR under conditions that increase the mis-incorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol.1, 1989, pp. 11-15). A mutator strain of E. coli (Fowler et al., Molec. Gen. Genet., 133, 1974, pp. 179-191), S. cerevisiae or any other microbial organism may be used for the random mutagenesis of the DNA encoding the alpha-amylase by, e.g., transforming a plasmid

containing the parent glycosylase into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may be subsequently transformed into the expression organism. The DNA sequence to be mutagenized may be conveniently present in a genomic or cDNA library prepared from an organism
5 expressing the parent alpha-amylase. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or otherwise exposed to the mutagenising agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harboured in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be
10 understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence. In some cases it may be convenient to amplify the mutated DNA sequence prior to performing the expression step b) or the screening step c). Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on
15 the basis of the DNA or amino acid sequence of the parent enzyme. Subsequent to the incubation with or exposure to the mutagenising agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA
20 sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are the following: gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans* or *Streptomyces murinus*; and gram-negative
25 bacteria such as *E. coli*. The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

Localised random mutagenesis

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

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

Alternative methods of providing alpha-amylase variants

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

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
25 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 that may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into
30 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
35 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 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 as *amdS*, *argB*, *niaD* and *sC*, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g., as described in WO 91/17243.

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

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

The cell of the invention, either comprising a DNA construct or an expression vector of the

invention as defined above, is advantageously used as a host cell in the recombinant production of an 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 methods, e.g., by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

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

Examples of suitable bacteria are 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*.

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 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.

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).

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, 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
5 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 purposes,
10 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.

15 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, and in the production of feed and food.

Starch Conversion

20 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.

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
30 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
35 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.

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 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

5

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.

10

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.

15

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 known as mash.

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Saccharification

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To produce low molecular sugars DP_{1-3} that can be metabolized by yeast, the maltodextrin from the liquefaction must be further hydrolyzed. The hydrolysis is typically done 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.

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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.

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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.

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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.

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By-products

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

15

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 carried out simultaneously or separately.

Pulp and Paper Production

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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 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:

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- 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).

30

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 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.

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Desizing of Textiles, Fabrics and Garments

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 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.

5 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
10 147 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
15 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®,
20 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
25 *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).

30 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).

35 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.

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

10 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 and WO 89/09259.

15 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 5,763,254, WO 95/24471, WO 98/12307 and PCT/DK98/00299.

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

25 Peroxidases/Oxidases: Suitable peroxidases/oxidases include those of plant, bacterial 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).

30 The detergent enzyme(s) may be included in a detergent composition by adding 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 20 (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, alpha-sulfo fatty acid 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 0.001-100 mg of enzyme protein per liter of wash liquor, preferably 0.005-5 mg of enzyme protein per liter of wash liquor, more preferably 0.01-1 mg of enzyme protein per liter of wash liquor and 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 Deterget 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%
Sodium disilicate	2 - 30%
Sodium carbonate	10 - 50%
Sodium phosphonate	0 - 5%
Trisodium citrate dehydrate	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%

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 $\text{KHSO}_5 \cdot \text{KHSO}_4 \cdot \text{K}_2\text{SO}_4$)	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

6) POWDER AND LIQUID DISHWASHING COMPOSITION WITH CLEANING SURFACTANT SYSTEM

Nonionic surfactant	0 - 1.5%
Octadecyl dimethylamine N-oxide dehydrate	0 - 5%
80:20 wt.C18/C16 blend of octadecyl dimethylamine N-oxide dihydrate and hexadecyldimethyl amine N-oxide dehydrate	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%

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 glycols, polyglycols, polyoxides and glycol ethers	Balance

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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-decyldiphenyl 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 dehydrate	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

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

13) 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.

MATERIALS AND METHODS

Enzymes:

SP722: SEQ ID NO: 4, available from Novozymes, and disclosed in WO 95/26397.

AA560: SEQ ID NO: 12; disclosed in WO 00/60060 and available from Novozymes A/S; disclosed in Danish patent application no. PA 1999 00490; deposited on 25th January 1999 at DSMZ and assigned the DSMZ no. 12649.

AA560 were deposited under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at Deutsche Sammlung von Microorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1b, D-38124 Braunschweig DE.

AX379: Available from Novozymes.

Bacillus subtilis SHA273: see WO 95/10603

Plasmids

pJE1 contains the gene encoding a variant of SP722 alpha-amylase (SEQ ID NO: 4): viz. deletion of 6 nucleotides corresponding to amino acids D183-G184 in the mature protein. Transcription of the JE1 gene is directed from the *amyL* promoter. The plasmid further more contains the origin of replication and *cat*-gene conferring resistance towards chloramphenicol obtained from plasmid pUB110 (Gryczan, TJ et al. (1978), J. Bact. 134:318-329).

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:

General molecular biology methods:

Unless otherwise mentioned the DNA manipulations and transformations were performed using standard methods of molecular biology (Sambrook et al. (1989); Ausubel et al. (1995); Harwood and Cutting (1990)).

Modelbuilding

Protein structure databases, such as "The Protein Data Bank (PDB) (<http://www.pdb.bnl.gov/>)" or "The Brookhaven databank at Brookhaven National Laboratory, US" are search for proteins similar to the molecule in question that a model are to be build of. The amino acid sequences are aligned taking structurally conserved regions into consideration and the coordinates are copied from the reference protein to the subject protein. The coordinates for regions with insertions and deletions are assigned either from other proteins having similar amino acid sequence, or by using the random structure generator function found in most 3D software packages, eg. in Homology from Biosym, MSI.

When coordinates have been assigned to all amino acids of the subjective protein and the fragments have been linked together, example by the cormands *END REPAIR* and *SPLICE REPAIR*, in the Discover program from from Biosym, MSI, the model are to be refined. The energy of the model is minimised first by relaxing the molecule (RELAX command in the Discover program) and second minimised by molecular dynamics.

References can be found in and in the manuals of homology building software, eg. Homology from Biosym, MSI

Method for obtaining the regions of interest:

There are three known 3D structures of bacterial α -amylases. Two of *B. licheniformis* α -amylase, Brookhaven database 1BPL (Machius et al. (1995), J. Mol. Biol. 246, p. 545-559) and 1VJS (Song et al. (1996), Enzymes for Carbohydrate 163 Engineering (Prog. Biotechnol. V 12). These two structures are lacking an important piece of the structure from the so-called B-domain, in the area around the two Calcium ions and one Sodium ion binding sites. There also exist a 3D structure of an alpha-amylase BA2 (WO 96/23874 which is a hybrid between BANTM (SEQ ID NO. 5) and *B. licheniformis* alpha-amylase (SEQ ID NO. 4) published, which contains the full B-domain and thus the metal ions between the A and B domain. Further a structure of the main part of the alpha-amylase from *B. stearothermophilus* has been published by Sued [?] and the structure of the alkaline alpha amylase SP722 was presented in WO 01/66712 .

To build the best model of a given alpha-amylase, the structure of the closed homolog is chosen, i.e. a good model of *B. licheniformis* alpha amylase is best build on basis of the structure of BA2, so is a good model of *B. amyloliquefaciens* alpha-amylase, while alkaline alpha-amylases like AA560, SP707, SP7-7 and KSM-AP1378 are best build on the structure of SP722 α -amylase.

Homology building of AA560 from SP722 tertiary structure

The overall homology of the AA560 alpha-amylase (SEQ ID NO: 12) to SP722 (SEQ ID NO: 4) is about 87% as described above. Sequence alignment of AA560 and SP722 shows where to be no insertion or deletions, which can also be seen in Fig. 1.

The tertiary structure of the AA560 alpha-amylase was model build on the structure disclosed in Appendix 1 using the method "Modelbuilding" described in the "Materials & Methods"-section.

The structure of SP722 was displayed on a UNIX work station running Insight and Homology software from BIOSYM, MSI. The amino acid sequences were aligned and the SP722 coordinated assigned to the AA560 amino acids. The coordinates of the first four amino acids in AA560, which are missing in the SP722 structure, were assigned by the "END REPAIR" function.

The AA560 model was refined by first relaxing the amino acid side chains, using the "RELAX" command and then running molecular dynamics to minimise the energy of the 3D model. Default parameters from Insight 95, MSI were chosen for both relaxation molecular dynamics.

Fermentation and purification of α -amylase variants

Fermentation and purification may be performed by methods well known in the art.

Fermentation of alpha-amylases and variants

5 Fermentation may be performed by methods well known in the art or as follows.

A *B. subtilis* strain harboring the relevant expression plasmid is streaked on a LB-agar plate with a relevant antibiotic, and grown overnight at 37°C.

The colonies are transferred to 100 ml BPX media supplemented with a relevant antibiotic (for instance 10 mg/l chloroamphenicol) in a 500 ml shaking flask.

10 Composition of BPX medium:

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

The culture is shaken at 37°C at 270 rpm for 4 to 5 days.

20 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 an 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 0.2 M NaCl in the same buffer. The eluate is dialysed against 10 mM Tris, pH 9.0 and applied on a Q-sepharose F.F. and eluted with a linear gradient from 0-
25 0.3M NaCl over 6 column volumes. The fractions, which contain the activity (measured by the Phadebas assay) are pooled, pH was adjusted to pH 7.5 and remaining color was removed by a treatment with 0.5% W/vol. active coal in 5 minutes.

Stability determination

30 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.

35 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 alpha-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.

Assays for Alpha-Amylase Activity1. Phadebas assay

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

For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM 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 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 (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.

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 substrate one bottle of substrate (BM 1442309) is added to 5 ml buffer (BM1442309). To prepare the α -Glucosidase one bottle of alpha-Glucosidase (BM 1462309) is added to 45 ml buffer (BM1442309). The working solution is made by mixing 5 ml alpha-Glucosidase solution with 1 ml substrate.

The assay is performed by transforming 20 μl enzyme solution to a 96 well microtitre plate and incubating at 25°C. 200 μl working solution, 25°C is added. The solution is mixed and pre-incubated 1 minute and absorption is measured every 15 sec. over 3 minutes at OD 405 nm.

The slope of the time dependent absorption-curve is directly proportional to the specific activity (activity per mg enzyme) of the alpha-amylase in question under the given set of conditions.

Specific activity determination

The specific activity is determined as activity/mg enzyme using one of the methods described above. The manufactures instructions are followed (see also below under "Assay for alpha-amylase activity).

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 by taking out samples and dilute them 5 times with ice-cold water and store them on ice.

The remaining activity is thus measured using the Phadebas method as described above where the absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the alpha-amylase activity. The activities after 5, 15 and 30 minutes are calculated relatively to the activity after 15sec., to determine the stability.

5

Washing Performance

Washing performance is evaluated by washing soiled test swatches for 15 and 30 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
10 above as described above in the Materials section) dosed from 3 to 5 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 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).

15

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

20

EXAMPLES

EXAMPLE 1

Construction of variants of AA560 SEQ ID NO: 12

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The gene encoding the AA560 alpha-amylase shown in SEQ ID NO: 12 is located in a plasmid pTVB223. The amylase is expressed from the amyL promoter in this construct in *Bacillus subtilis*.

A variant of the invention with M202L mutation was constructed by the mega-primer method as described by Sarkar and Sommer, (1990), BioTechniques 8: 404-407.

30

The resulting plasmid encoding the AX379 amylase with M202L was named pCA202-AX379

The construction of the other variants of the invention was carried out in a similar manner.

35

EXAMPLE 2Determination of activity in wash

Amylase variants were constructed using conventional methods in the amylase AX379 or AX413, respectively which in the activity test is used as reference (first line in the table).

The AX413 variant is derived from AX379 by introducing mutations as indicated in the tables below.

The activity was measured in detergent solution in a simulated European wash at 40°C. A suspension of one Phadebas tablet per 5ml of 4g/L detergent solution was made and adequated under stirring into Eppendorf tubes. After 5 minutes of pre-heating at 40°C Amylase enzyme was add and the mixture incubated for 20 min under vigorous shaking. The reaction is stopped by adding 10% 1M NaOH and the tubes are spin for 3 min at 10000xg as minimum. Finally the absorbance at 650nm is measured for the supernatant using a non-enzyme sample as blind.

Table 3:

Mutations	Improvement in activity
AX379	1
K118Q	1,1
K37T	1,1
H421Y	1,1
V450T	1,1
K383R	1,1
N445Q	1,1
Y178F	1,2
V318L	1,2
W482Y	1,2
N283D+Q361E	1,2
M105F+M208F	1,2
M202L+M323T+M430I	1,3
K446R+N484Q	1,4
R444Y	1,5
N106D	1,8
Y203L	2,0
G133E+Q361E	2,9
M323E	4,1
V214T	6,8

M202L+M323T+M309L	13
M202L	16
M202L+M323T	23
M202L+M323T+M9L+M382Y+K383R	25
M202L+M323T+M9L+M382Y	26
M202L+M323T+M9L (AX413)	27

Table 4:

Mutations	Improvement in activity
AX413	1
T461P	1,1
Y298H	1,1
G133E+R444E	1,1
Y298F	1,1
M202T	1,2
M202I	1,6
M202V	1,6
Y295F	3,4

5 EXAMPLE 3

Determination of stability during dishwash

Amylase variants were constructed using conventional methods in the amylase AX379 or AX413, respectively which in the activity test is used as reference (first line in the table). The AX413 variant is derived from AX379 by introducing mutations as indicated in the tables above.

The amylase stability was measured by incubating around 0.1 mg/ml amylase in 4 g/l detergent for automatic dishwash at 40°C for 18 hours. The incubation was stopped by adding 9 volumes of cold (<5°C) water and stored on ice. With one hour the activity was measured using the Phadebas Amylase kit and the activity in detergent samples compared to samples incubated for the same period in detergent but on ice.

Table 5:

Mutations	Improvement in residual activity
AX413	1
V214T+M323E+M382Y+K383R+N471E	1.1

Y178F+G258D+T419N+N437H	1.1
G149N+N150Q+M382Y+K383R	1.1
Y160F+V214T+M382Y	1.2
N128Y+G149A+V214T+D231N+M382Y+F441L	1.2
R82H+N128Y+G149A+V214T+M382Y	1.2
N150H+V214T	1.2
V214T+E345N	1.2
V214T+G305D+ M382Y+R444E	1.2
V214T+M382Y+A447Y	1.2
M202I+V214T+M382Y+K383R+R444Y	1.2
V214T+G378K	1.3
V214T+A256K	1.3
R26S+D30N+N33D+V214T+M382Y	1.5

EXAMPLE 4

Amylase variants of seq. ID no. 12 were constructed as described in example 1 and fermented in shakeflasks using a rich media. From the supernatant the amylase variant protein was purified using standard purification methods to above 90% purity. The protein concentration was calculated from A280 absorbance and a theoretic extension coefficient of 2.9 ml/mg/cm.

The G7-pNP activity assay was used as described under "Methods" to measure the activity of the amylase samples and thus the specific activity (SA), i.e. the G7-pNP activity per mg of amylase protein was calculated and compared to a homologous reference amylase.

	Rel. SA
M202L (Ref.A)	1,00
Ref.A+M9L+M323T	1,18
Ref.A+M9L+M323T+M382Y+K383R	1,20
Ref.A+M9L+S303Q+M323T+M382Y+K383R	1,31
Ref.A+M9L+V214T+M323T+M382Y	1,37
Ref.A+M9L+M323T+A339S+M382Y+K383R+N471E	1,55
Ref.A+M9L+V214T+M323T+A339S+N471E	1,73

	Rel. SA
M202L+V214T (Ref. B)	1,00
Ref.B+G149H	1,41
Ref.B+E345R	1,18
Ref.B+G149A+M382Y	1,24
Ref.B+G149A+N299Y+T356I+M382Y	1,27
Ref.B+M382Y	1,43
Ref.B+G149A+K269S+N270Y+Y295F+A339S+E345R+N471E	1,78
Ref.B+A339S+N471E	1,80

	Rel. SA
M9L+M202I+M323T (Ref. C)	1,00
Ref.C+V214T+Y295F+A339S+M382Y+K383R+N471E	2,60

	Rel. SA
M9L+M202I+V214T+Y295F+M323T+M382Y (Ref.D)	1,00
Ref.D+G149A+A339S+E345R	1,25
Ref.D+G149A+V214I+A339S	1,25
Ref.D+N83S+G149A+A339S+E345R	1,25
Ref.D+G133K+G149A+A339S+E345R	1,33
Ref.D+I206F+A339S	1,42
Ref.D+G149A+A339S	1,50
Ref.D+G149A+V214V+K269S+N270Y+E345R+A339S	4,08
Ref.D+G149A+V214V+K269S+N270Y+A339S	5,83

5 EXAMPLE 5

Wash tests were conducted using 9 g/l (Henkel) HDD traditional detergent with bleach and 0.2 mg amylase protein per litre in a down scaled washing machine, applying a general European heat-up profile to 40°C over 20 minutes. The water hardness is adjusted with Ca, Mg and NaHCO₃ to 16°dH.

10 The washing performance is evaluated on cotton swatches with colored rice starch, (CS-28 from CFT), by measuring the whiteness of the swatch after wash with amylase present relative to the whiteness of a swatch washed without amylase. The whiteness is measured using a remission spectrophotometer (Macbeth Color-Eye), after the swatches have dried on

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lines over night.

/

	IF
AX379	1,00
M9L+G149A+M202I+V214T+Y295F+M323T+A339S+E345R+M382Y	1,06
G149A+G182T+G186A+M202I+V214I+Y295F+N299Y+M323T+A339S	1,12
M9L+G149A+M202I+V214I+Y295F+M323T+A339S+M382Y	1,13
M9L+N106D+M202L+M323T	1,14
M9L+M202L+M323T+M382Y+K383R	1,14
M9L+M202L+V214T+Y295F+M323T+M382Y	1,14
M9L+G133K+G149A+M202I+V214T+Y295F+M323T+A339S+E345R+M382Y	1,14
M9L+M202I+V214T+M323T+A339S+M382Y+K383R+N471E	1,16
M9L+M202L+V214T+M323T+M382Y	1,17
M9L+G149A+M202L+V214T+M323T+M382Y	1,17
M9L+M202L+S303Q+M323T+M382Y+K383R	1,17
M9L+G149A+G182T+M202L+T257I+Y295F+S303Y+M323T+A339S+E345R	1,17
M9L+G149A+M202L+V214T+N299Y+M323T+T356I+M382Y	1,18
M9L+G149H+M202L+V214T+M323T	1,20
M9L+M202L+V214T+M323T+E345R	1,25
M9L+G149A+G182T+M202L+T257I+Y295F+N299Y+M323T+A339S+E345R	1,25
M9L+G149A+M202L+V214I+Y295F+M323T+A339S	

	1,26
M9L+G149A+M202L+V214I+Y295F+M323T+A339S+E345R	1,30
M9L+M202L+V214T+Y295F+M323T+A339S	1,31
M9L+G149A+G182T+G186A+M202L+T257I+Y295F+N299Y+M323T+A339S+E345R	1,35
M9L+M202L+M323T+A339S+M382Y+K383R+N471E	1,38

EXAMPLE 6

Wash tests were conducted using 6 g/l Persil Megaperls (Henkel) detergent and 0.2 mg
 5 amylase protein per litre in a down scaled washing machine, applying a general European
 heat-up profile to 40°C over 20 minutes. The water hardness is adjusted with Ca, Mg and
 NaHCO₃ to 16°dH.

The washing performance is evaluated on cotton swatches with colored rice starch, (CS-
 28 from CFT), by measuring the whiteness of the swatch after wash with amylase present
 10 relative to the whiteness of a swatch washed without amylase. The whiteness is measured
 using a remission spectrophotometer (Macbeth Color-Eye), after the swatches have dried on
 lines over night.

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 52

NOTE : Pour les tomes additionels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 52

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

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 26, 30, 33, 82, 37, 106, 118, 128, 133, 149, 150, 160, 178, 182, 186, 193, 203, 214, 231, 256, 257, 258, 269, 270, 272, 283, 295, 296, 298, 299, 303, 304, 305, 311, 314, 315, 318, 319, 339, 345, 361, 378, 383, 419, 421, 437, 441, 444, 445, 446, 447, 450, 461, 471, 482, 484, wherein

(a) the alteration(s) are independently

10 (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

15 (c) each position corresponds to a position of the amino acid sequence of the parent alpha-amylase having the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid sequence of AA560 shown in SEQ ID NO: 12.

2. The variant of claim 1, wherein the mutations are:

20 R26S, D30N, N33D, R82H, K37T, N106D, K118Q, N128Y, G133E,A, G149A,N, N150H,Q, Y160F, Y178F, G182T, G186A, T193S,N,D,E,Q, Y203L, V214I,T, D231N, G256K, T257I, G258D, K269S,Q, N270F,Y,D, L272I,V,A, N283D, Y295F,N,D,Q,E, N296K,Q,E, Y298F,H, N299F,Y,Q,T, S303Q,K, Y304F,R,K, G305D, Q311N,Q,K,R,T,S,Y,F, N314D,S,T,Q, G315N,S,T, V318L, Q319E,K,S,T, A339S,T, E345N,R, Q361E, G378K, K383R, T419N, 25 H421Y, N437H, F441L, R444E,Y, N445Q, K446R, A447Y, V450T, T461P, N471E, W482Y, N484Q.

3. The variant of claims 1-2, wherein the variant has an additional mutation in one or more methionine residues.

4. The variant of claim 3, wherein the methionine residues are:

M9, M10, M116, M202, M208, M261, M309, M323, M382, M410, M430, and M440.

5. The variant of claim 4, wherein the mutations are:

35 M9L,I, M10L, M105L,I,F, M116N,D,L,I,F,W,R,K, M202I,L,V,T, M208F,Y,L,I, M261L,I, M309L,I, M323L,I,S,T,A,Q,E,N,D, M382L,I,Y,F,K, M410L,I,V, M430L,I, and M440L,I,F,Y.

6. The variant of claims 1-5, wherein the variant has one or more of the following mutations:

M9L+M202I,

M9L+M202I+M323T,

M9L+M202I+323T+M382Y,

5 M9L+M202I+Y295F+A339S,

M9L+M202I+Y295F,

M9L+M202I+A339S,

M9L+M202I+Y295F+A339S,

M9L+M202I+Y295F+A339S+E345R,

10 M9L+G149A+M202I+Y295F+A339S+E345R,

M9L+M202L,

M9L+M202L+M323T,

M9L+M202L+M232T+M382Y,

M9L+M202L+Y295F+A339S,

15 M9L+M202L+Y295F,

M9L+M202L+A339S,

M9L+M202L+Y295F+A339S,

M9L+M202L+Y295F+A339S, E345R,

M9L+G149A+M202L+Y295F+A339S+E345R,

20 M9L+M202T,

M9L+M202T+M323T,

M9L+M202T+M323T+M382Y,

M9L+M202T+Y295F+A339S,

M9L+M202T+Y295F,

25 M9L+M202T+A339S,

M9L+M202T+Y295F+A339S,

M9L+M202T+Y295F+A339S+E345R,

M9L+G149A+M202T+Y295F+A339S+E345R,

M9L+G149A+M202I+V214T+Y295F+N299Y+M323T+A339S+E345R,

30 M9L+G149A+M202L+V214I+Y295F+M323T+A339S+E345R+M382Y,

M9L+G149A+G182T+G186A+M202I+V214I+Y295F+N299Y+M323T+A339S,

M9L+G149A+G182T+G186A+M202L+T257I+Y295F+N299Y+M323T+A339S+E345R,

M9L+G149A+M202L+V214T+Y295F+N299Y+M323T+A339S+E345R,

M9L+G149A+M202I+V214I+Y295F+M323T+A339S+E345R+M382Y,

35 M9L+G149A+G182T+G186A+M202L+V214I+Y295F+N299Y+M323T+A339S,

M9L+G149A+G182T+G186A+M202I+T257I+Y295F+N299Y+M323T+A339S+E345R,

M9L+G149A+M202I+V214T+Y295F+N299Y+M323T+A339S+E345R+N471E,

M9L+G149A+M202L+V214I+Y295F+M323T+A339S+E345R+M382Y+N471E,
M9L+G149A+G182T+G186A+M202I+V214I+Y295F+N299Y+M323T+A339S+N471E,
M9L+G149A+G182T+G186A+M202L+T257I+Y295F+N299Y+M323T+A339S+E345R+N471E,
M202L+M105F+M208F,
5 G133E+M202L+Q361E,
G133E+M202L+R444E,
M202L+Y295F,
M202L+A339S,
M202L+M323T,
10 M202L+M323T+M309L,
M202L+M323T+M430I,
M202L+V214T+R444Y,
M202L+N283D+Q361E,
M202L+M382Y+K383R,
15 M202L+K446R+N484Q,
M202I+Y295F,
M202I+A339S,
M202I+M105F+M208F,
G133E+M202I+Q361E,
20 G133E+M202I+R444E,
M202I+M202I+M323T,
M202I+M202I+M323T+M309L,
M202I+M323T+M430I,
M202I+V214T+R444Y,
25 M202I+N283D+Q361E,
M202I+M382Y+K383R,
M202I+K446R+N484Q,
M202V+M105F+M208F,
G133E+M202V+Q361E,
30 G133E+M202V+R444E,
M202V+M323T,
M202V+M323T+M309L,
M202V+M323T+M430I,
M202V+M323T+M9L,
35 M202V+V214T+R444Y,
M202V+N283D+Q361E,
M202V+M382Y+K383R,

M202V+K446R+N484Q,
 M202T+M105F+M208F,
 G133E+M202T+Q361E,
 G133E+M202T+R444E,
 5 M202T+Y295F,
 M202T+A339S,
 M202T+M323T,
 M202T+M323T+M309L,
 M202T+M323T+M430I,
 10 M202T+M323T+M9L,
 M202T+V214T+R444Y,
 M202T+N283D+Q361E,
 M202T+A339S,
 M202T+Y295F
 15 M202T+N299F,Y,
 M202T+M382Y+K383R,
 M202T+K446R+N484Q

- 20 7. The variant of claims 1-6, wherein the variant further comprises the mutation D183*+G184*.
8. The variant of claims 1-7, wherein the variant further comprises a mutation in R118, in particular R118K.
- 25 9. The variant of claims 1-8, wherein the variant further comprises a mutation in N195, in particular N195F.
10. The variant of claims 1-9, wherein the variant further comprises a mutation in R320, in particular R320K.
- 30 11. The variant of claims 1-10, wherein the variant further comprises a mutation in R458, in particular R458K.
12. The variant of claims 1-11, wherein the variant comprises the mutation D183*+G184*+R118K+N195F+R320K+R458K in combination with one or more of the following mutations:
- 35 K118Q,

K37T,
 H421Y,
 V450T,
 K383R,
 N445Q,
 Y178F,
 V318L,
 W482Y,
 N283D+Q361E,
 M105F+M208F,
 M202L+M323T+M430I,
 K446R+N484Q,
 R444Y,
 N106D,
 Y203L,
 G133E+Q361E,
 M323E,
 V214T,
 M202L+M323T+M309L,
 M202L,
 M202L+M323T,
 M202L+M323T+M9L+M382Y+K383R,
 M202L+M323T+M9L+M382Y,
 M202L+M323T+M9L.

13. The variant of claim 12, wherein the variant comprises the mutation D183*+G184*+R118K+N195F+R320K+R458K+M202L+M323T+M9L.

5 14. The variant of claim 13 wherein the variant further comprises one or more of the following mutations:

T461P,
 Y298H,
 G133E+R444E,
 Y298F,
 M202T,
 M202I,
 M202V,
 V214T+M323E+M382Y+K383R+N471E
 Y178F+G258D+T419N+N437H
 G149N+N150Q+M382Y+K383R
 Y160F+V214T+M382Y
 N128Y+G149A+V214T+D231N+M382Y+F441L
 R82H+N128Y+G149A+V214T+M382Y
 N150H+V214T
 V214T+E345N
 V214T+G305D+ M382Y+R444E
 V214T+M382Y+A447Y
 M202I+V214T+M382Y+K383R+R444Y
 V214T+G378K
 V214T+A256K
 R26S+D30N+N33D+V214T+M382Y

15. The variant of any of claims 1-14, wherein the parent Termamyl-like alpha-amylase is derived from a strain of *B. licheniformis*, *B. amyloliquefaciens*, *B. stearothermophilus*, *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, or DSMZ no. 12649, KSM AP1378, or KSM K36 or KSM K38.

16. The variant of claims 1-15, wherein the parent Termamyl-like alpha-amylase is any of the alpha-amylases selected from the group depicted in SEQ ID NOS: 2, 4, 6, 8, 10, 12, 13, 14, 15 16, 17, and 18.

17. The variant according to any of claims 1-16, wherein the parent Termamyl-like alpha-amylase has an amino acid sequence which has a degree of identity to SEQ ID NO: 4 of at least 60%, preferably 70%, more preferably at least 80%, even more preferably at least about 90%, even more preferably at least 95%, even more preferably at least 97%, and even more preferably at least 99%.

18. The variant of any of claims 1-17, wherein the parent Termamyl-like alpha-amylase is encoded by a nucleic acid sequence, which hybridizes under low, preferably medium, preferred high stringency conditions, with the nucleic acid sequence of SEQ ID NO: 11.

5

19. The variant of claims 1-18, which variant has 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 pI and improved wash
10 performance, specific activity, stability under, e.g., high temperature and/or low/high pH conditions, in particular at low calcium concentrations, and stability in the presence of detergent, e.g. storage stability in the detergents.

15

20. A DNA construct comprising a DNA sequence encoding an alpha-amylase variant according to any one of claims 1 to 19.

21. A recombinant expression vector which carries a DNA construct according to claim 20.

20

22. A cell which is transformed with a DNA construct according to claim 20 or a vector according to claim 21.

25

23. A cell according to claim 22, which is a microorganism, preferably a bacterium or a fungus, in particular a gram-positive bacterium, such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*,
25 *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus* or *Bacillus thuringiensis*.

24. A composition comprising an alpha-amylase variant of claims 1-19.

30

25. A detergent additive comprising an alpha-amylase variant according to any one of claims 1 to 19, optionally in the form of a non-dusting granulate, stabilised liquid or protected enzyme.

26. A detergent additive according to claim 25, which contains 0.02-200 mg of enzyme protein/g of the additive.

35

27. A detergent additive according to claims 25 or 26, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, mannanase, maltogenic amylase, CGTase, amylase or another amylolytic enzyme, such as glucoamylase, and/or a cellulase.

28. A detergent composition comprising an alpha-amylase variant according to any of claims 1 to 19.

5 29. A detergent composition according to claim 28, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, mannanase, maltogenic amylase, CGTase, another amylolytic enzyme and/or a cellulase.

10 30. A manual or automatic dishwashing detergent composition comprising an alpha-amylase variant according to any of claims 1 to 19.

31. A dishwashing detergent composition according to claim 30, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, mannanase, maltogenic amylase, CGTase, amylase or another amylolytic enzyme, such as glucoamylase, and/or a cellulase.

15 32. A manual or automatic laundry washing composition comprising an alpha-amylase variant according to any of claims 1 to 19.

20 33. A laundry washing composition according to claim 32, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, mannanase, maltogenic amylase, CGTase, an amylase and/or another an amylolytic enzyme, such as glucoamylase and/or a cellulase.

34. Use of an alpha-amylase variant according to any one of claims 1 to 19 or a composition according to claims 24 to 34 for washing and/or dishwashing.

25 35. Use of an alpha-amylase variant according to any one of claims 1 to 19 or a composition according to claims 24 to 34 for textile desizing.

30 36. Use of an alpha-amylase variant according to any of claims 1 to 19 or a composition according to claims 24 to 34 for starch liquefaction.

37. A method of producing a variant according to any of claims 1-19, wherein a cell according to any of claims 22 to 23 is cultured under conditions conducive to the production of the variant, and the variant is subsequently recovered from the culture.

1/3

		1	50
	BLA	(1)	--ANLNGTLMQYFEWYMPNDGQHWKRLQND SAYLAEHGITAVWI PPAYKG
	AMY1048	(1)	-SVPVNGTMMQYFEWYLPDDGTLWTKVANNAQSLANLGITALWL PPAYKG
	BSG	(1)	-AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWL PPAYKG
5	BAN	(1)	---AVNGTLMQYFEWYTPNDGQHWKRLQND AEHLSDIGITAVWI PPAYKG
	B.sp.K38	(1)	--DGLNGTMMQYFEWHLNDGQHWNRHLHDDAAALSDAGITAIWI PPAYKG
	AAI-10	(1)	HHNGTNGTIMQYFEWNPNDGQHWNRHLHNAQNLKNAGITAIWI PPAYKG
	SP722	(1)	HHNGTNGTMMQYFEWHL PNDGNHWNRLRDDASNLRNRGITAIWI PPAYKG
	SP690	(1)	HHNGTNGTMMQYFEWYLPNDGNHWNRLRDDAANLKS KGITAVWI PPAYKG
10	AA560	(1)	HHNGTNGTMMQYFEWYLPNDGNHWNRLRSDASNLDKDGISAVWI PPAYKG
	Amrk385	(1)	HHNGTNGTMMQYFEWHL PNDGQHWNRRLRND AANLKNLGITAVWI PPAYKG
	B.sp.707	(1)	HHNGTNGTMMQYFEWYLPNDGNHWNRLNSDASNLDKSKGITAVWI PPAYKG
	B.sp.7-7	(1)	HHNGTNGTMMQYFEWYLPNDGNHWNRLRSDASNLDKDGITAVWI PPAYKG
	KSM-AP1378	(1)	HHNGTNGTMMQYFEWHL PNDGNHWNRLRDDAANLKS KGITAVWI PPAYKG
15		51	100
	BLA	(49)	TSQADVGYGAYDLYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVY
	AMY1048	(50)	TSSSDVGYGVYDLYDLGEFNQKGTVRTKYGKTQYIQAIQAAHTAGMQVY
	BSG	(50)	TSRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVY
20	BAN	(48)	LSQSDNGYGPYDLYDLGEFQQKGTVRTKYGTKSELQDAIGSLHSRNVQVY
	B.sp.K38	(49)	NSQADVGYGAYDLYDLGEFNQKGTVRTKYGTKAQLERAIGSLKSNDINVY
	AAI-10	(51)	TSQNDVGYGAYDLYDLGEFNQKGTVRTKYGTKAELERAIRSLKANGIQVY
	SP722	(51)	TSQNDVGYGAYDLYDLGEFNQKGTVRTKYGTRSQLESALHALKNNGVQVY
	SP690	(51)	TSQNDVGYGAYDLYDLGEFNQKGTVRTKYGTRNQLQAAVTS LKNNGIQVY
25	AA560	(51)	ASQNDVGYGAYDLYDLGEFNQKGTIRTKYGTRNQLQAAVNALKSNGIQVY
	Amrk385	(51)	TSQNDVGYGAYDLYDLGEFNQKGTIRTKYGTRS QLSAIALQNNNGIQVY
	B.sp.707	(51)	ASQNDVGYGAYDLYDLGEFNQKGTVRTKYGTRS QLSAIALQNNNGIQVY
	B.sp.7-7	(51)	ASQNDVGYGAYDLYDLGEFNQKGTVRTKYGTRNQLQAAVTALKNNGIQVY
	KSM-AP1378	(51)	TSQNDVGYGAYDLYDLGEFNQKGTVRTKYGTRS QLSAIALQNNNGIQVY
30		101	150
	BLA	(99)	GDVVINHKG GADATEDVTAVEVDPADRNRVISGEHRIKAWTHFHF PGRGS
	AMY1048	(100)	ADVVFNHKAGADGTELVD AVEVNPSDRNQEISGTYQIQAWTKFDF PGRGN
	BSG	(100)	ADVVFHDKG GADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDF PGRGN
35	BAN	(98)	GDVVLNHKAGADATEDVTAVEVN PANRNQETSEYQIKAWTDFRFPGRGN
	B.sp.K38	(99)	GDVVMNHKMGADFT EAVQAVQVNPTNRWQDISGAYTIDAWTG FDFSGRNN
	AAI-10	(101)	GDVVMNHKG GADFTERVQAVEVNPNRNQEVSGTYQIEAWTG FNF PGRGN
	SP722	(101)	GDVVMNHKG GADATENVLAVEVNPNRNQEVSGDYTIEAWTK FDF PGRGN
	SP690	(101)	GDVVMNHKG GADGTEIVNAVEVNRSNRNQETS GEYAIEAWTKFDF PGRGN
40	AA560	(101)	GDVVMNHKG GADATEMVR AVEVNPNRNQEVSGEYTIEAWTK FDF PGRGN
	Amrk385	(101)	GDVVMNHKG GADGTEWVQAVEVNPSNRNQEVTGEYTIEAWTK FDF PGRGN
	B.sp.707	(101)	GDVVMNHKG GADATEMVR AVEVNPNRNQEVSGEYTIEAWTR FDF PGRGN
	B.sp.7-7	(101)	GDVVMNHKG GADATEMVR AVEVNPSNRNQEVS GDYTIEAWTR FDF PGRGN
	KSM-AP1378	(101)	GDVVMNHKG GADGTEMVNAVEVNRSNRNQEIS GEYTIEAWTK FDF PGRGN
45		151	200
	BLA	(149)	TYSDFKWHWYHFDGTDWDESR-KLNRIYKFQG--KAWDWEVSNENGNYDY
	AMY1048	(150)	TYSSFKWRWYHFDGTDWDESR-KLNRIYKFRGTGKAWDWEVDTENGNYDY
	BSG	(150)	TYSSFKWRWYHFDGVDWDESR-KLSRIYKFRGIGKAWDWEVDTENGNYDY
50	BAN	(148)	TYSDFKWHWYHFDGADWDESR-KISRIYKFRGEGKAWDWEVSSENGNYDY
	B.sp.K38	(149)	AYSDFKWRWFHFN GVDWDQRY-QENHIFRFAN--TNWNWRVDEENGNYDY
	AAI-10	(151)	QHSSFKWRWYHFDGTDWDQSRQLANRIYKFRGDGKAWDWEVDTENGNYDY
	SP722	(151)	TYSDFKWRWYHFDGVDWDQSRQFQNR IYKFRGDGKAWDWEVDS ENGNYDY
	SP690	(151)	NHSSFKWRWYHFDGTDWDQSRQLQNKIYKFRGTGKAWDWEVDTENGNYDY
55	AA560	(151)	THSNFKWRWYHFDGVDWDQSRKLNNRIYKFRGDGKGWDWEVDTENGNYDY
	Amrk385	(151)	THSSFKWRWYHFDGTDWDQSRQLNNRIYKFRGTGKAWDWEVDTENGNYDY
	B.sp.707	(151)	THSSFKWRWYHFDGVDWDQSRRLNNRIYKFRGHGKAWDWEVDTENGNYDY
	B.sp.7-7	(151)	THSNFKWRWYHFDGVDWDQSRQLQNR IYKFRGDGKAWDWEVDTENGNYDY
	KSM-AP1378	(151)	THSNFKWRWYHFDGTDWDQSRQLQNKIYKFRGTGKAWDWEVDIENGNYDY
60			

Fig. 1

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		201	250
	BLA	(196)	LMYADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKFSFRLRDWV
	AMY1048	(199)	LMYADLDMDHPEVVSELKNWGKWYVTTTNIDGFRLDAVKHIKYSFFPDWL
5	BSG	(199)	LMYADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFPPDWL
	BAN	(197)	LMYADVDDYDHPDVVAETKKWGIWYANELSLDGFRLDAAKHIKFSFRLRDWV
	B.sp.K38	(196)	LLGSNIDFSHPEVQDELKDWSWFTDELDLDGYRLDAIKHIPFWYTSDWV
	AAI-10	(201)	LMYADVDMMDHPEVINELNRWGVWYANTLNLDGFRLDAVKHIKFSFMRDWL
	SP722	(201)	LMYADVDMMDHPEVVNELRRWGEWYTNTLNLDGFRLDAVKHIKYSFTRDWL
10	SP690	(201)	LMYADVDMMDHPEVIHELNRWGVWYNTNTLNLDGFRLDAVKHIKYSFTRDWL
	AA560	(201)	LMYADIDMDHPEVVNELNRWGVWYNTNLGLDGFRLDAVKHIKYSFTRDWI
	Amrk385	(201)	LMYADVDMMDHPEVINELRRWGVWYNTNLNLDGFRLDAVKHIKYSFTRDWL
	B.sp.707	(201)	LMYADIDMDHPEVVNELNRWGVWYNTNLGLDGFRLDAVKHIKYSFTRDWI
	B.sp.7-7	(201)	LMYADIDMDHPEVVNELNRWGVWYNTNLGLDGFRLGAVKHIKYSFTRDWL
15	KSM-AP1378	(201)	LMYADIDMDHPEVINELNRWGVWYNTNLNLDGFRLDAVKHIKYSYTRDWL
		251	300
	BLA	(246)	NHVREKTGKE-MFTVAEYWQNDLGALENYLNKTNFNHVSFVDVPLHYQFHA
	AMY1048	(249)	SYVRTQTQKP-LFAVGEFWSYDISKLHNYITKTNGSMSLFDAPLHNNFYI
20	BSG	(249)	SYVRSQTGKP-LFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYT
	BAN	(247)	QAVRQATGKE-MFTVAEYWQNNAGKLENYLNKTSFNQSVFVDVPLHFNLQA
	B.sp.K38	(246)	RHQRNEADQD-LFVVGEYWKDDVGALEFYLDENWEMSLFDVPLNLYNFYR
	AAI-10	(251)	GHVRGQTGKN-LFAVAEYWKNDLGALENYLSKTNWTMSAFDVPLHYNLYQ
	SP722	(251)	THVRNATGKE-MFAVAEFWKNDLGALENYLNKTNWNHVSFVDVPLHYNLYN
25	SP690	(251)	THVRNTTGKP-MFAVAEFWKNDLGALENYLNKTSWNHVSFVDVPLHYNLYN
	AA560	(251)	NHVRSATGKN-MFAVAEFWKNDLGALENYLNKTNWNHVSFVDVPLHYNLYN
	Amrk385	(251)	NHVRSTTGKNMFVAEYWKNDLGALENYLNKTNWNHVSFVDVPLHYNLYN
	B.sp.707	(251)	NHVRSATGKN-MFAVAEFWKNDLGALENYLQKTNWNHVSFVDVPLHYNLYN
	B.sp.7-7	(251)	THVRNTTGKN-MFAVAEFWKNDIGAILENYLSKTNWNHVSFVDVPLHYNLYN
30	KSM-AP1378	(251)	THVRNTTGKP-MFAVAEFWKNDLAAILENYLNKTSWNHVSFVDVPLHYNLYN
		301	350
	BLA	(295)	ASTQGGGYDMRKLNLNGTVVSKHPLKAVTFVDNHDTPGQSLESTVQTFWK
	AMY1048	(298)	ASKSGGYFDMRTLNLNNTLMKDQPTLAVTLVDNHDTEPGQSLQSWVEPWFK
35	BSG	(298)	ASKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFK
	BAN	(296)	ASSQGGGYDMRRLLDGTVVSRHPEKAVTFVENHDTQPGQSLESTVQTFWK
	B.sp.K38	(295)	ASQGGGSYDMRNILRGSLVEAHPMHAVTFVDNHDTPGESLESWVADWFK
	AAI-10	(300)	ASNSSGNYDMRNLLNGTLVQRHPHAVTFVDNHDTPGGEALESFVQGWFK
	SP722	(300)	ASNSSGNYDMAKLLNGTVVQKHPMHAVTFVDNHDSPGESLESFVQEWFK
40	SP690	(300)	ASNSSGNYDMRNILNGSVVQKHPHAVTFVDNHDSPGESLESFVQQWFK
	AA560	(300)	ASKSGGNYDMRQIFNGTVVQRHPMHAVTFVDNHDSPPEEALESFVEEWFK
	Amrk385	(301)	ASKSGGNYDMRQILNGTVVSKHPIHAVTFVDNHDSPGESLESFVEAWFK
	B.sp.707	(300)	ASKSGGNYDMRNIFNGTVVQRHPHAVTFVDNHDSPPEEALESFVEEWFK
	B.sp.7-7	(300)	ASRSSGNYDMRQIFNGTVVQRHPHAVTFVDNHDSPPEEALESFVEEWFK
45	KSM-AP1378	(300)	ASNSSGNYFDMRNILNGSVVQKHPHAVTFVDNHDSPGESLESFVQSWFK
		351	400
	BLA	(345)	PLAYAFILTRESGYPQVFYGDYMGTKGDSQREIPALKHKIEPILKARKQY
	AMY1048	(348)	PLAYAFILTRQEGYPCVFYGDYYGIPKYN---IPALKSKLDPLLIARRDY
50	BSG	(348)	PLAYAFILTRQEGYPCVFYGDYYGIPQYN---IPSLKSKIDPLLIARRDY
	BAN	(346)	PLAYAFILTRESGYPQVFYGDYMGTKGTSPKEIPSLKDNIEPILKARKEY
	B.sp.K38	(345)	PLAYATILTREGGYPNVFYGDYYGIPNDN---ISAKKDMIDELLDARQNY
	AAI-10	(350)	PLAYATILTREQGYPCVFYGDYYGIPSDG---VPSYRQQIDPLLKARQQY
	SP722	(350)	PLAYALILTREQGYPSVFYGDYYGIPTHS---VPAMKAKIDPILEARQNF
55	SP690	(350)	PLAYALVLTREQGYPSVFYGDYYGIPTHG---VPAMKSKIDPLLQARQTF
	AA560	(350)	PLAYALTLTREQGYPSVFYGDYYGIPTHG---VPAMKSKIDPILEARQKY
	Amrk385	(351)	PLAYALILTREQGYPSVFYGDYYGIPTHG---VAAMKGKIDPILEARQKY
	B.sp.707	(350)	PLAYALTLTREQGYPSVFYGDYYGIPTHG---VPAMRSKIDPILEARQKY
	B.sp.7-7	(350)	PLACALTLTRDQGYPSVFYGDYYGIPTHG---VPAMKSKIDPILEARQKY
60	KSM-AP1378	(350)	PLAYALILTREQGYPSVFYGDYYGIPTHG---VPSMKSKIDPLLQARQTY
		401	450
	BLA	(395)	AYGAQHDIYFDHHDIVGWTREGDSSVANSGLAALITDGPGGAKRMYVGRQN
65	AMY1048	(395)	AYGTQHDYIDSADIIGWTREGVAEKANSGLAALITDGPGGSKWYVVGKQH

Fig. 1 (continued)

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	BSG	(395)	AYGTQHDYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQH	
	BAN	(396)	AYGPQHDYIDHPDVIIGWTREGDSSAAKSGLAALITDGPGGSKRMYAGLKN	
	B.sp.K38	(392)	AYGTQHDYFDHWDVVGWTREGSSSRPNNSGLATIMSDGPGGSKWMYVGRQN	
5	AAI-10	(397)	AYGRQHDYFDHWDVIGWTREGNASHPNNSGLATIMSDGPGGSKWMYVGRQK	
	SP722	(397)	AYGTQHDYFDHHDIIIGWTREGNTTHPNNSGLATIMSDGPGGSKWMYVGQNK	
	SP690	(397)	AYGTQHDYFDHHDIIIGWTREGNSSHPNSGLATIMSDGPGGSKWMYVGKKN	
	AA560	(397)	AYGRQNDYLDHHDIIIGWTREGNTAHPNSGLATIMSDGAGGSKWMFVGRNK	
	Amrk385	(398)	AYGTQHDYLDHHDIIIGWTREGNSAHPNSGLATIMSDGPGGSKWMYVGRHK	
10	B.sp.707	(397)	AYGKQNDYLDHHDIIIGWTREGNTAHPNSGLATIMSDGAGGSKWMFVGRNK	
	B.sp.7-7	(397)	AYGKQNDYLDHHDIIIGWTREGNTAHPNSGLATIMSDGPGGSKWMYVGRNK	
	KSM-AP1378	(397)	AYGTQHDYFDHHDIIIGWTREGDSSHNSGLATIMSDGPGGSKWMYVGKHK	
			451	500
15	BLA	(445)	AGETWHDITGNRSEPVVINSEGWGEFHVNGGSVSIYVQR-----	
	AMY1048	(445)	AGKTFYDLTGNRSDTVTINADGWGEFVKVNGGSVSIWVPKISTTSQITFTV	
	BSG	(445)	AGKVIFYDLTGNRSDTVTINSDGWGEFVKVNGGSVSVWVPRKTTVSTIARPI	
	BAN	(446)	AGETWYDITGNRSDTVKIGSDGWGEFHVNDGSVSIYVQK-----	
	B.sp.K38	(442)	AGQWTDLTGNGASVTINGDGWGEFFTNNGGSVSVYVNQ-----	
20	AAI-10	(447)	AGEVWHDMTGNRSGTVTINQDGWGHFFVNGGSVSVWVKR-----	
	SP722	(447)	AGQVWHDITGNKPGTVTINADGWANFSVNGGSVSIWVKR-----	
	SP690	(447)	AGQVWRDITGNRTGTVTINADGWGNFSVNGGSVSVWVKQ-----	
	AA560	(447)	AGQVWTDITGNRAGTVTINADGWGNFSVNGGSVSIWVNK-----	
	Amrk385	(448)	AGQVWRDITGNRTGTVTINADGWGNFSVNGGSVSIWVNK-----	
25	B.sp.707	(447)	AGQVWSDITGNRTGTVTINADGWGNFSVNGGSVSIWVNK-----	
	B.sp.7-7	(447)	AGQVWRDITGNRSGTVTINADGWGNFSVNGGSVSIWVNN-----	
	KSM-AP1378	(447)	AGQVWRDITGNRSGTVTINADGWGNFTVNGGAVSVWVKQ-----	
			501	550
30	BLA	(484)	-----	
	AMY1048	(495)	NNATTVWGQNVYVVGNI SQLGNWDPVHAVQMTTPSSYPTWTVTIPLLOGQN	
	BSG	(495)	TTRPWTG---EFVRWTEPRLVAWP-----	
	BAN	(485)	-----	
	B.sp.K38	(481)	-----	
35	AAI-10	(486)	-----	
	SP722	(486)	-----	
	SP690	(486)	-----	
	AA560	(486)	-----	
	Amrk385	(487)	-----	
40	B.sp.707	(486)	-----	
	B.sp.7-7	(486)	-----	
	KSM-AP1378	(486)	-----	
			551	591
45	BLA	(484)	-----	
	AMY1048	(545)	IQFKFIKKDSAGNVIWEDISNRTYTVPTAASGAYTASWNVP	
	BSG	(516)	-----	
	BAN	(485)	-----	
	B.sp.K38	(481)	-----	
50	AAI-10	(486)	-----	
	SP722	(486)	-----	
	SP690	(486)	-----	
	AA560	(486)	-----	
	Amrk385	(487)	-----	
55	B.sp.707	(486)	-----	
	B.sp.7-7	(486)	-----	
	KSM-AP1378	(486)	-----	

Fig. 1 (continued)