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(54) Titre : MUTANTS D'ALPHA-AMYLASE A PROPRIETES MODIFIEES
(54) Title: ALPHA-AMYLASE MUTANTS WITH ALTERED PROPERTIES

(57) Abrégé/Abstract:
The present invention relates to variants (mutants) of parent Termamyl-like alpha-amylases, which variant has alpha-amylase activity and exhibits altered stability, in particular at high temperatures and/or low pH relative, and/or low Ca^{2+} to the parent alpha-amylase.

ABSTRACT

The present invention relates to variants (mutants) of parent Termamyl-like alpha-amylases, which variant has alpha-amylase activity and exhibits altered stability, in particular at high temperatures and/or low pH relative, and/or low Ca2+ to the parent alpha-amylase.

Alpha-Amylase Mutants With Altered Properties

CROSS REFERENCE TO RELATED APPLICATION

This application is a divisional of application No. 2,416,967 filed on July 12, 2001.

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: stability under, e.g., high temperature and/or low pH conditions, in particular at low calcium concentrations. The variant of the invention are suitable for starch conversion, ethanol production, laundry wash, dish wash, hard surface cleaning, textile desizing, and/or sweetner production.

BACKGROUND OF THE INVENTION

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

BRIEF DISCLOSURE OF THE INVENTION

The object of the present invention is to provide 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 following properties relative to said parent alpha-amylase: stability under, e.g., high temperature and/or low pH conditions, in particular at low calcium concentrations.

Nomenclature

In the present description and claims, the conventional one-letter and three-letter codes for amino acid residues are

used. For ease of reference, alpha-amylase variants of the invention are described by use of the following nomenclature:
Original amino acid(s): position(s): substituted amino acid(s)

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

Ala30Asn or A30N

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

Ala30* or A30*

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

Ala30AlaLys or A30AK

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

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

*36Asp or *36D

for insertion of an aspartic acid in position 36.
Multiple mutations are separated by plus signs, i.e.:

Ala30Asp + Glu34Ser or A30N+E34S

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

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

A30N,E or

A30N or A30E

Furthermore, when a position suitable for modification is identified herein without any specific modification being suggested, it is to be understood that any amino acid residue may be substituted for the amino acid residue present in the position. Thus, for instance, when a modification of an alanine in position 30 is mentioned, but not specified, it is to be understood that the alanine may be deleted or substituted for any other amino acid, i.e., any one of: R,N,D,A,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

Further, "A30X" means any one of the following substitutions: A30R, A30N, A30D, A30C, A30Q, A30E, A30G, A30H, A30I, A30L, A30K, A30M, A30F, A30P, A30S, A30T, A30W, A30Y, or A30 V; or in short: A30R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

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

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

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

BRIEF DESCRIPTION OF THE DRAWINGS

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

1: SEQ ID NO: 4 (SP722)

2: SEQ ID NO: 2 (SP690)

3: SEQ ID NO: 10 (BAN)

4: SEQ ID NO: 8 (BLA)

5: SEQ ID NO: 6 (BSG).

DETAILED DISCLOSURE OF THE INVENTION

The object of the present invention is to provide Termamyl-like amylases, which variants have alpha-amylase activity and exhibits altered stability at high temperatures and/or at low pH, in particular at low calcium concentrations.

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 known Bacillus alpha-amylases can be found in the below Table 1:

Table 1

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

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

The KSM AP1378 alpha-amylase is disclosed in WO 97/00324 (from KAO Corporation).

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

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

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

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

Homology

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

A structural alignment between Termamyl (SEQ ID NO: 8) and, e.g., another alpha-amylase may be used to identify equivalent/corresponding positions in other Termamyl-like alpha-amylases. One method of obtaining said structural alignment is to use the Pile Up programme from the GCG package using default values of gap penalties, i.e., a gap creation penalty of 3.0 and gap extension penalty of 0.1. Other structural alignment methods include the hydrophobic cluster analysis (Gaboriaud et al., (1987), FEBS LETTERS 224, pp. 149-155) and reverse threading (Huber, T; Torda, AE, PROTEIN SCIENCE Vol. 7, No. 1 pp. 142-149 (1998).

Hybridisation

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

Suitable conditions for testing hybridisation involve pre-soaking in 5xSSC and 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 100 mM ATP for 18 hours at 40°C, followed by three times washing of the filter in 2xSSC, 0.2% SDS at 40°C for 30 minutes (low stringency), preferred at 50°C (medium stringency), more preferably at 65°C (high stringency), even more preferably at 75°C (very high stringency). More details about the hybridisation method can be found in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989.

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

Parent Termamyl-like Alpha-amylases

According to the invention all Termamy-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: 8.

Parent hybrid Termamyl-like Alpha-amylases

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

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

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

For instance, the parent alpha-amylase may comprise a C-terminal part of an alpha-amylase derived from a strain of B. licheniformis, and a N-terminal part of an alpha-amylase derived from a strain of B. amyloliquefaciens or from a strain of B. stearothermophilus. For instance, the parent alpha-amylase may comprise at least 430 amino acid residues of the C-terminal part of the B. licheniformis alpha-amylase, and may, e.g., comprise a) an amino acid segment corresponding to the 37 N-terminal amino acid residues of the B. amyloliquefaciens alpha-amylase having the amino acid sequence shown in SEQ ID NO: 10 and an amino acid segment corresponding to the 445 C-terminal amino acid residues of the B. licheniformis alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8, or a hybrid Termamyl-like alpha-amylase being identical to the Termamyl sequence, i.e., the Bacillus licheniformis alpha-amylase shown in SEQ ID NO: 8, except that the N-terminal 35 amino acid residues (of the mature protein) has been replaced by the N-terminal 33 residues of BAN (mature protein), i.e., the Bacillus amyloliquefaciens alpha-amylase shown in SEQ ID NO: 10; or b) an amino acid segment corresponding to the 68 N-terminal amino acid residues of the B. stearothermophilus alpha-amylase having the amino acid sequence shown in SEQ ID NO: 6 and an amino acid segment corresponding to the 415 C-terminal amino acid residues of the B. licheniformis alpha-amylase having the amino acid sequence shown in SEQ ID NO: 8.

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

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

Other specifically contemplated parent alpha-amylase include LE174 with fewer mutations, i.e., the right above mentioned hydrid having the following mutations: A181T+N190F+A209V+Q264S; N190F+A209V+Q264S; A209V+Q264S; Q264S; H156Y+N190F+A209V+Q264S; H156Y+A209V+Q264S; H156Y+Q264S; H156Y+A181T+A209V+Q264S; H156Y+A181T+Q264S; H156Y+Q264S; H156Y+A181T+N190F+Q264S; H156Y+A181T+N190F; H156Y+A181T+N190F+A209V. These hybrids are also considered to be part of the invention.

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

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.

Altered properties

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

As mentioned above the invention relates to Termamyl-like alpha-amylases with altered properties (as mentioned above), 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 70-120°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 Termamyl® alpha-amylase is used as the starting point, but corresponding positions in, e.g., the SP722, BSG, BAN, AA560, SP690, KSM AP1378, and #707 should be understood as disclosed and specifically comtemplated too.

In a preferred embodiment the variant of the invention has in particular at high temperatures and/or at low pH.

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

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

49, 60, 104, 132, 161, 170, 176, 179, 180, 181, 183, 200, 203, 204, 207, 212, 237, 239, 250, 280, 298, 318, 374, 385, 393, 402, 406, 427, 430, 440, 444, 447, 482,

wherein

(a) the alteration(s) are independently

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

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

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

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

In Termamyl® (SEQ ID NO: 8) such corresponding positions are:

T49; D60; N104; E132; D161; K170; K176; G179; K180; A181; D183; D200; Y203; D204; D207; I212; K237; S239; E250; N280; Q298; L318; Q374; E385; Q393; Y402; H406; L427 D430; V440; N444; E447; Q482.

In SP722 (SEQ ID NO: 4) the corresponding positions are: T51; D62; N106; D134; D163; Q172; K179; G184; K185; A186; D188; D205; M208; D209; X212; L217, K242, S244, N255, N285, S303, M323; D387, N395; Y404; H408; I429; D432; V442; K446; Q449; K484.

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

In a preferred embodiment the variant of the invention (using SEQ ID NO: 8 (Termamyl™) for the numbering) has one or more of the following substitutions:
T49I; D60N; N104D; E132A,V,P; D161N; K170Q; K176R; G179N; K180T; A181N; D183N; D200N; X203Y; D204S; D207V,E,L,G; X212I; K237P; S239W; E250G,F; N280S; X298Q; L318M; Q374R; E385V; Q393R; Y402F; H406L,W; L427I D430N; V440A; N444R,K; E447Q,K; Q482K.

In a preferred embodiment the variant of the invention (using SEQ ID NO: 4 (SP722) for the numbering) has one or more of the following substitutions:
T51I; D62N; N106D; D134A,V,P; D163N; X172Q; K179R; G184N; K185T; A186N; D188N; D205N; M208Y; D209S; X212V,E,L,G; L217I, K242P, S244W, N255G,F, N285S, S303Q, X323M; D387V, N395R; Y404F; H408L,W; X429I; D432N; V442A; X446R,K; X449Q,K; X484K, using SEQ ID NO: 4 (SP722) for the numbering.

Preferred double, triple and multi-mutations - using SEQ ID NO: 8 as the basis for the numbering - are selected from the group consisting of:
T49I+D60N; T49I+D60N+E132A; T49I+D60N+E132V;
T49I+D60N+E132V+K170Q; T49I+D60N+E132A+K170Q;
T49I+D60N+E132V+K170Q+K176R; T49I+D60N+E132A+K170Q+K176R;
T49I+D60N+E132V+K170Q+K176R+D207V;
T49I+D60N+E132A+K170Q+K176R+D207V;
T49I+D60N+E132V+K170Q+K176R+D207E;
T49I+D60N+E132A+K170Q+K176R+D207E;

T49I+D60N+E132V+K170Q+K176R+D207V+E250G;

T49I+D60N+E132A+K170Q+K176R+D207V+E250G;

T49I+D60N+E132V+K170Q+K176R+D207E+E250G;

T49I+D60N+E132A+K170Q+K176R+D207E+E250G;

T49I+D60N+E132V+K170Q+K176R+D207E+E250G+N280S;

T49I+D60N+E132A+K170Q+K176R+D207E+E250G+N280S;

T49I+D60N+E132V+K170Q+K176R+D207V+E250G+N280S;

T49I+D60N+E132A+K170Q+K176R+D207V+E250G+N280S;

T49I+D60N+E132V+K170Q+K176R+D207V+E250G+N280S+L318M;

T49I+D60N+E132A+K170Q+K176R+D207V+E250G+N280S+L318M;

T49I+D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M;

T49I+D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M;

T49I+D60N+E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R;

T49I+D60N+E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R;

T49I+D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R;

T49I+D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R;

T49I+D60N+E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V;

T49I+D60N+E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V;

T49I+D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V;

T49I+D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V;

T49I+D60N+E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R;

T49I+D60N+E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R;

T49I+D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R;

T49I+D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+ Q393R;

T49I+D60N+E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F;

T49I+D60N+E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F;

T49I+D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F;

T49I+D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+ Q393R+Y402F;

T49I+D60N+E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F+H406L;

T49I+D60N+E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L;

T49I+D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L;

T49I+D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+ Q393R+Y402F+H406L;

T49I+D60N+E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F+H406L+L427I;

T49I+D60N+E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I;

T49I+D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I;

T49I+D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+ Q393R+Y402F+H406L+L427I;

T49I+D60N+E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F+H406L+L427I+V440A;

T49I+D60N+E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A;

T49I+D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A;

T49I+D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+ Q393R+Y402F+H406L+L427I+V440A;

D60N+E132A; D60N+E132V; D60N+E132V+K170Q; D60N+E132A+K170Q; D60N+E132V+K170Q+K176R; T49I+D60N+E132A+K170Q+K176R; D60N+E132V+K170Q+K176R+D207V; T49I+D60N+E132A+K170Q+K176R+D207V; D60N+E132V+K170Q+K176R+D207E; T49I+D60N+E132A+K170Q+K176R+D207E; D60N+E132V+K170Q+K176R+D207V+E250G; D60N+E132A+K170Q+K176R+D207V+E250G; D60N+E132V+K170Q+K176R+D207E+E250G; D60N+E132A+K170Q+K176R+D207E+E250G; D60N+E132V+K170Q+K176R+D207V+E250G+N280S; D60N+E132A+K170Q+K176R+D207V+E250G+N280S; D60N+E132V+K170Q+K176R+D207E+E250G+N280S; D60N+E132A+K170Q+K176R+D207E+E250G+N280S; D60N+E132V+K170Q+K176R+D207V+E250G+N280S+L318M; D60N+E132A+K170Q+K176R+D207V+E250G+N280S+L318M; D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M; D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M; D60N+E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R; D60N+E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R; D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R; D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R; D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V; D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V; D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V; D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V; D60N+E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+ E385V+Q393R+Y402F; D60N+E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+ E385V+Q393R+Y402F; D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+ E385V+Q393R+Y402F;

D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F;
D60N+E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F+H406L;
D60N+E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L;
D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L;
D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L;
D60N+E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F+H406L+L427I;
D60N+E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I;
D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I;
D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I;
D60N+E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F+H406L+L427I+V440A;
D60N+E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A;
D60N+E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A;
D60N+E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A;
E132V+K170Q; E132A+K170Q; E132V+K170Q+K176R; E132A+K170Q+K176R;
E132V+K170Q+K176R+D207V; E132A+K170Q+K176R+D207V;
E132V+K170Q+K176R+D207E; E132A+K170Q+K176R+D207E;
E132V+K170Q+K176R+D207V+E250G; E132A+K170Q+K176R+D207V+E250G;
E132V+K170Q+K176R+D207E+E250G; E132A+K170Q+K176R+D207E+E250G;

E132V+K170Q+K176R+D207E+E250G+N280S;
E132A+K170Q+K176R+D207E+E250G+N280S;
E132V+K170Q+K176R+D207V+E250G+N280S;
E132A+K170Q+K176R+D207V+E250G+N280S;
E132V+K170Q+K176R+D207V+E250G+N280S+L318M;
E132A+K170Q+K176R+D207V+E250G+N280S+L318M;
E132V+K170Q+K176R+D207E+E250G+N280S+L318M;
E132A+K170Q+K176R+D207E+E250G+N280S+L318M;
E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R;
E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R;
E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R;
E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R;
E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V;
E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V;
E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V;
E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V;
E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R;
E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R;
E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R;
E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R;
E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F;
E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F;
E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F;
E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F;
E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F+H406L;
E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L;

E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L; E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L; E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F+H406L+L427I; E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I; E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I; E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I; E132V+K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F+H406L+L427I+V440A; E132A+K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A; E132V+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A; E132A+K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A; K170Q+K176R; K170Q+K176R+D207V; K170Q+K176R+D207E; K170Q+K176R+D207V+E250G; K170Q+K176R+D207E+E250G; K170Q+K176R+D207V+E250G+N280S; K170Q+K176R+D207E+E250G+N280S; K170Q+K176R+D207E+E250G+N280S+L318M; K170Q+K176R+D207V+E250G+N280S+L318M; K170Q+K176R+D207E+E250G+N280S+L318M+Q374R; K170Q+K176R+D207V+E250G+N280S+L318M+Q374R; K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V; K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V; K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R; K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R; K170Q+K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F; K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F;

K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+385V+Q393R+Y402F+H406L;

K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L;

K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F+H406L+L427I;

K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I;

K170Q+K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F+H406L+L427I+V440A;

K170Q+K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A;

K176R+D207V; K176R+D207E; K176R+D207V+E250G;

K176R+D207E+E250G;K176R+D207V+E250G+N280S;

K176R+D207E+E250G+N280S;K176R+D207E+E250G+N280S+L318M;

K176R+D207V+E250G+N280S+L318M;

K176R+D207E+E250G+N280S+L318M+Q374R;

K176R+D207V+E250G+N280S+L318M+Q374R;

K176R+D207E+E250G+N280S+L318M+Q374R+E385V;

K176R+D207V+E250G+N280S+L318M+Q374R+E385V;

K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R;

K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R;

K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F;

K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F;

K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L;

K176R+D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I;

K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I;

K176R+D207V+E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F+H406L+L427I+V440A;

K176R+D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A;

D207V+E250G; D207E+E250G;
D207V+E250G+N280S; D207E+E250G+N280S+L318M;
D207V+E250G+N280S+L318M;D207E+E250G+N280S+L318M+Q374R;
D207V+E250G+N280S+L318M+Q374R;
D207E+E250G+N280S+L318M+Q374R+E385V;
D207V+E250G+N280S+L318M+Q374R+E385V;
D207V+E250G+N280S+L318M+Q374R+E385V+Q393R;
D207E+E250G+N280S+L318M+Q374R+E385V+Q393R;
D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F;
D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F;
D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L;
D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L;
D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I;
D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I;
D207V+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A;
D207E+E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A; E250G+N280S; E250G+N280S+L318M;
E250G+N280S+L318M+Q374R;
E250G+N280S+L318M+Q374R+E385V;
E250G+N280S+L318M+Q374R+E385V+Q393R;
E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F;
E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L;
E250G+N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I;
E250G+N280S+L318M+Q373R+E385V+Q393R+Y402F+H406L+L427I+V440A;
N280S+L318M; N280S+L318M+Q374R; N280S+L318M+Q374R+E385V;
N280S+L318M+Q374R+E385V+Q393R;
N280S+L318M+Q374R+E385V+Q393R+Y402F;
N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L;
N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I;
N280S+L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A;
L318M+Q374R; L318M+Q374R+E385V; L318M+Q374R+E385V+Q393R;

L318M+Q374R+E385V+Q393R+Y402F; L318M+Q374R+E385V+Q393R+Y402F+H406L; L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I; L318M+Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A; Q374R+E385V; Q374R+E385V+Q393R; Q374R+E385V+Q393R+Y402F; Q374R+E385V+Q393R+Y402F+H406L; Q374R+E385V+Q393R+Y402F+H406L+L427I; Q374R+E385V+Q393R+Y402F+H406L+L427I+V440A; E385V+Q393R; E385V+Q393R+Y402F; E385V+Q393R+Y402F+H406L; E385V+Q393R+Y402F+H406L+L427I; E385V+Q393R+Y402F+H406L+L427I+V440A; Q393R+Y402F; Q393R+Y402F+H406L; Q393R+Y402F+H406L+L427I; Q393R+Y402F+H406L+L427I+V440A; Y402F+H406L; Y402F+H406L+L427I; Y402F+H406L+L427I+V440A; H406L+L427I; H406L+L427I+V440A; L427I+V440A; N104D+D161N+G179N+K180T+A181N+D183N+D200N+D204S+K237P+S239W+H406W+D430N+N444K+E447Q+Q482K; D161N+G179N+K180T+A181N+D183N+D200N+D204S+K237P+S239W+H406W+D430N+N444K+E447Q+Q482K; D161N+A181N+D183N+D200N+D204S+K237P+S239W+H406W+D430N+N444K+E447Q+Q482K; D161N+A181N+D183N+D200N+D204S+K237P+S239W+H406W+D430N+E447Q+Q482K; N104D+D161N+G179N+K180T+A181N+D183N+D200N+D204S+K237P+S239W+H406W+D430N+E447Q+Q482K; D161N+G179N+K180T+A181N+D183N+D200N+D204S+K237P+S239W+H406W+D430N+E447Q+Q482K; N104D+D161N+G179N+K180T+A181N+D183N+D200N+D204S+K237P+S239W+H406W+D430N; D161N+G179N+K180T+A181N+D183N+D200N+D204S+K237P+S239W+H406W+D430N; H406W+D430N; N444K+E447Q+Q482K; E447Q+Q482K; N104D+D161N+G179N+K180T+A181N+D183N+D200N+D204S+K237P+S239W+

H406W+D430N+N444R+N444K+E447K+Q482K; D161N+G179N+K180T+A181N+D183N+D200N+D204S+K237P+S239W+H406W+D430N+N444R+N444K+E447K+Q482K; N104D+D161N+G179N+K180T+A181N+D183N+D200N+D204S+K237P+S239W; D161N+G179N+K180T+A181N+D183N+D200N+D204S+K237P+S239W; H406W+D430N; N444K+E447K+Q482K; E447K+Q482K; N104D+D161N+A181N+D183N+D200N+D204S+K237P+S239W; N104D+D161N+A181N+D183N+D200N+D204S+K237P; N104D+D161N+A181N+D183N+D200N+D204S; D161N+A181N+D183N+D200N+D204S+K237P+S239W; D161N+A181N+D183N+D200N+D204S+K237P; D161N+A181N+D183N+D200N+D204S; K237P+S239W, using SEQ ID NO: 8 for the numbering.

In a preferred embodiment the variant has the following substitutions: K170Q+D207V+N280S; E132A+D207V; D207E+E250G+H406L+L427I; D207V+L318M; D60N+D207V+L318M; T49I+E132V+V440A; T49I+K176R+D207V+Y402F; Q374R+E385V+Q393R; N190F+A209V+Q264S; G48A+T49I+G107A+I201F; T49I+G107A+I201F; G48A+T49I+I201F; G48A+T49I+G107A; T49I+I201F; T49I+G107A; G48A+T49I; D161N+G179N+K180T+A181N+D183N+D200N+D204S+K237P+S239W+H406W+D430N+N444K+E447Q+Q482K using SEQ ID NO: 8 for the numbering. Specific variant include: LE399; LE174+G48A+T49I+G107A; LE174+G48A+T49I+I201F; LE174+G48A+G107A+I201F; LE174+T49I+G107A+I201F; LE174+G48A+T49I; LE174+G48A; LE174+G107A+I201F; LE174+I201F, are specifically contemplated variants of the invention.

Stability

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

General mutations in variants of the invention

A variant of the invention may in one embodiment comprise one or more modifications in addition to those outlined above. Thus, it may be advantageous that one or more Proline (Pro) residues present in the part of the alpha-amylase variant which is modified is/are replaced with a non-Proline residue which may be any of the possible, naturally occurring non-Proline residues, and which preferably is an Alanine, Glycine, Serine, Threonine, Valine or Leucine.

Analogously, in one embodiment one or more Cysteine residues present in the parent alpha-amylase may be replaced with a non-Cysteine residue such as Serine, Alanine, Threonine, Glycine, Valine or Leucine.

Furthermore, a variant of the invention may - either as the only modification or in combination with any of the above outlined modifications - be modified so that one or more Asp and/or Glu present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID NO: 10 is replaced by an Asn and/or Gln, respectively. Also of interest is the replacement, in the Termamyl-like alpha-amylase, of one or more of the Lys residues present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID NO: 10 by an Arg.

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

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

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

M15X, in particular M15T,L;
V128X, in particular V128E;
H133X, in particular H133Y;
N188X, in particular N188S,T,P;
M197X, in particular M197T,L;
A209X, in particular A209V;
M197T/W138F; M197T/W138Y; M15T/H133Y/N188S;
M15/V128E/H133Y/N188S; E119C/S130C; D124C/R127C; H133Y/T149I;
G475R, H133Y/S187D; H133Y/A209V.

Methods for preparing alpha-amylase variants of the invention

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

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. Examples of parent alpha-amylases, which suitably may be used for providing a hybrid with the desired mutations(s) according to the invention include the KSM-K36 and KSM-K38 alpha-amylases disclosed in EP 1,022,334.

Expression of alpha-amylase variants

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

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

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

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

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids 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 alpha-amylases mentioned herein comprise a preregion permitting secretion of the expressed protease into the culture medium. If desirable, this preregion may be replaced by a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention encoding an 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 gramnegative 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 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 publications Nos. 252 730 and 63 909, WO 99/19467, and WO 96/28567). Also contemplated are compositions for starch conversion purposes, which may beside the variant of the invention also comprise a AMG, 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.

A variant of the invention may also be used for textile desizing (see, e.g., WO 95/21247, US patent 4,643,736, EP 119,920).

Detergent compositions

As mentioned above, variants of the invention may suitably be incorporated in detergent compositions. Reference is made, for example, to WO 96/23874 and WO 97/07202 for further details concerning relevant ingredients of detergent compositions (such as laundry or dishwashing detergents), appropriate methods of formulating the variants in such detergent compositions, and for examples of relevant types of detergent compositions.

Detergent compositions comprising a variant of the invention may additionally comprise one or more other enzymes, such as a protease, a lipase, a peroxidase, another amylolytic enzyme, glucoamylase, maltogenic amylase, CGTase and/or a cellulase, mannanase (such as Mannaway™ from Novozymes, Denmark)), pectinase, pectine lyase, cutinase, laccase, and/or another alpha-amylase.

Alpha-amylase variants of the invention may be incorporated in detergents at conventionally employed concentrations. It is at present contemplated that a variant of the invention may be incorporated in an amount corresponding to 0.00001-10 mg (calculated as pure, active enzyme protein) of alpha-amylase per liter of wash/dishwash liquor using conventional dosing levels of detergent.

Compositions

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

In another embodient the composition comprises beside a variant of the invention a glucoamylase, in particular a glucoamylase originating from Aspergillus niger (e.g., the G1 or G2 A. niger AMG disclosed in Boel et al. (1984), "Glucoamylases G1 and G2 from Aspergillus niger are synthesized from two different but closely related mRNAs", EMBO J. 3 (5), p. 1097-1102, or a variant therefore, in particular a variant disclosed in WO 00/04136 or WO 01/04273 or the Talaromyces emersonii AMG disclosed in WO 99/28448.

A specific combination is LE399 and a variant disclosed in WO 00/04136 or Wo 01/04273, in particular a variant with oe or more of the following substitutions: N9A,S56A,V59A,S119P,A246T,N313G,E342T,A393R,S394R,Y402F,E408R, in particular a variant with all mutation.

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

MATERIALS AND METHODS

Enzymes:

Bacillus licheniformis alpha-amylase shown in SEQ ID NO: 8 and also available from Novozymes.

AA560: SEQ ID NO: 12; disclosed in WO 00/60060; deposited on 25th January 1999 at DSMZ and assigned the DSMZ no. 12649. AA560 were deposited by the inventors under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at Deutshe Sammmlung von Microorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1b, D-38124 Braunschweig DE.

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

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

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

Bacillus subtilis SHA273: see WO 95/10603

Plasmids

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

Methods:

Low pH filter assay

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

Each filter sandwich is specifically marked with a needle after plating, but before incubation in order to be able to localize positive variants on the filter, and the nitrocellulose filter with bound variants is transferred to a container with citrate buffer, pH 4.5 and incubated at 80°C for 20 minutes (when screening for variants in the wild type backbone) or 85oC for 60 minutes (when screening for variants in the LE399 backbone). The cellulose acetate filters with colonies are stored on the TY-plates at room temperature until use. After incubation, residual activity is detected on assay plates containing 1% agarose, 0.2% starch in citrate buffer, pH 6.0. The assay plates with nitrocellulose filters are marked the same way as the filter sandwich and incubated for 2 hours at 50°C. After removal of the filters the assay plates are stained with 10% Lugol solution. Starch degrading variants are detected as white spots on dark blue background and then identified on the storage plates. Positive variants are re-screened twice under the same conditions as the first screen.

Secondary screening

Positive transformants after rescreening are picked from the storage plate and tested in a secondary plate assay. Positive transformants are grown for 22 hours at 37°C in 5 ml LB + chloramphenicol. The Bacillus culture of each positive transformant and as a control a clone expressing the corresponding backbone are incubated in citrate buffer, pH 4.5 at 90°C and samples are taken at 0, 10, 20, 30, 40, 60 and 80 minutes. A 3 micro liter sample is spotted on an assay plate.

The assay plate is stained with 10% Lugol solution. Improved variants are seen as variants with higher residual activity (detected as halos on the assay plate) than the backbone. The improved variants are determined by nucleotide sequencing.

Stability assay of unpurified variants:

Bacillus cultures expressing the variants to be analysed are grown for 21 hours at 37°C in 10 ml LB+chloramphenicol. 800 micro liter culture is mixed with 200 micro l citrate buffer, pH 4.5. A number of 70 micro l aliquots corresponding to the number of sample time points are made in PCR tubes and incubated at 70°C (for variants in the wt backbone) or 90°C (for variants in LE399) for various time points (typically 5, 10, 15, 20, 25 and 30 minutes) in a PCR machine. The 0 min sample is not incubated at high temperature. Activity in the sample is measured by transferring 20 micro l to 200 micro l of the alpha-amylase PNP-G7 substrate MPR3 ((Boehringer Mannheim Cat. no. 1660730) as described below under "Assays for Alpha-Amylase Activity". Results are plotted as percentage activity (relative to the 0 time point) versus time, or stated as percentage residual activity after incubation for a certain period of time.

Fermentation and purification of alpha-amylase variants

A B. subtilis strain harbouring the relevant expression plasmid is streaked on a LB-agar plate with 10 micro g/ml kanamycin from -80°C stock, and grown overnight at 37°C.
The colonies are transferred to 100 ml PS-1 media supplemented with 10 micro g/ml chloamphinicol in a 500 ml shaking flask.

Composition of PS-1 medium:

Pearl sugar	100 g/l
Soy Bean Meal	40 g/l
Na2HPO4, 12 H2O	10 g/l
PluronicTM PE 6100	0.1 g/l
CaCO3	5 g/l

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

Cells and cell debris are removed from the fermentation broth by centrifugation at 4500 rpm in 20-25 minutes. Afterwards the supernatant is filtered to obtain a completely clear solution. The filtrate is concentrated and washed on a UF-filter (10000 cut off membrane) and the buffer is changed to 20mM Acetate pH 5.5. The UF-filtrate is applied on a S-sepharose F.F. and elution is carried out by step elution with 0.2M NaCl in the same buffer. The eluate is dialysed against 10mM Tris, pH 9.0 and applied on a Q-sepharose F.F. and eluted with a linear gradient from 0-0.3M NaCl over 6 column volumes. The fractions that contain the activity (measured by the Phadebas™ assay) are pooled, pH was adjusted to pH 7.5 and remaining color was removed by a treatment with 0.5% W/vol. active coal in 5 minutes.

Stability determination of purified variants

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

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

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

Specific activity determination

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

Assays for Alpha-Amylase Activity

1. 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 spectophometry at λ=405nm (400-420 nm). Kits containing PNP-G7 substrate and alpha-Glucosidase is manufactured by Boehringer-Mannheim (cat. No.1054635).

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

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

EXAMPLES

Example 1.

Construction, by error-prone PCR mutagenesis, of Bacillus licheniformis alpha-amylase variants having an improved stability at low pH, high temperature and low calcium ion concentration compared to the parent enzyme.

Error-prone PCR mutagenesis and library construction

To improve the stability at low pH and low calcium concentration of the parent Bacillus licheniformis alpha-amylase, error-prone PCR mutagenesis was performed. The plasmid pDN1528 encoding the wild-type Bacillus licheniformis alpha-amylase gene was utilized as template to amplify this gene with primers: 22149: 5'-CGA TTG CTG ACG CTG TTA TTT GCG-3' (SEQID NO: 14) and 24814: 5'-GAT CAC CCG CGA TAC CGT C-3' (SEQ ID NO: 15) under PCR conditions where increased error rates leads to introduction of random point mutations. The PCR conditions utilized were: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 4 mM MgCl2, 0.3 mM MnCl2, 0.1mM dGTP/dATP, 0.5 mM dTTP/dCTP, and 2.5 units Taq polymerase per 100 micro l reaction.

The resultant PCR fragment was purified on gel and used in a PCR-based multimerization step with a gel purified vector fragment created by PCR amplification of pDN1528 with primers #24: 5'-GAA TGT ATG TCG GCC GGC AAA ACG CCG GTG A-3' (SEQ ID NO: 16) and #27: 5'-GCC GCC GCT GCT GCA GAA TGA GGC AGC AAG-3' (SEQ ID NO:17) forming an overlap to the insert fragment. The multimerization reaction was subsequently introduced into B. subtilis (Shafikhani et al., Biotechniques, 23 (1997), 304-310).

Screening

The error-prone library described above was screened in the low pH filter assay (see "Materials & Methods"). Clones testing positive upon rescreening was submitted to secondary screening for stability in the liquid assay described in Materials and Methods.

Results:

Increased stability at pH 4.5, 5 ppm calcium incubated at 90°C

Name	wt	LE488	LE489	7.19.1	8.9.1
Mutations	-	D207V	K170Q D207V N280S	E132A D207V	D207E E250G H406L L427I
Stability1)	-	+	+	+	+

1) A "+" indicates significant increase in stability relative to wild type.

Increased stability at pH 4.5, 5 ppm calcium incubated at 90°C

Name	wt	LE491	LE492	LE493	LE494	19.3.1
Mutations	-	D60N D207V L318M	T49I E132V V440A	T49I K176R D207V Y402F	Q374R E385V Q393R	N190F A209V Q264S
Stability1)	-	+	+	+	+	+

1) A "+" indicates significant increase in stability relative to wt.

Increased stability at pH 4.5, 5 ppm calcium incubated at 90°C

Name	wt	E132-1	D207-7	D207-6	E250-8
Mutations	-	E132P	D207L	D207G	E250F
Stability1)	-	+	+	+	+

1) A "+" indicates significant increase in stability relative to wt.

Example 2

Transfer, by site-directed mutagenesis, of a selection of mutations from Example 1 to a new (non-wild type) backbone to improve stability at low pH and low calcium ion concentration compared to the parent enzyme.

Site-directed mutagenesis

Mutations from LE493 (K176R+D207V+Y402F) were transferred to LE399 yielding LE495. This was performed by the overlap PCR method (Kirchhoff and Desrosiers, PCR Methods and Applications, 2 (1993), 301-304). 2 overlapping PCR fragments were generated by amplification of the LE399 template with the primers: Fragment A: #312 Mut176 5'-CCC GAA AGC TGA ACC GCA TCT ATA GGT TTC AAG GGA AGA CTT GGG ATT-3' (SEQ ID NO: 18)(mutated codon indicated in bold) and #290 D207overlap 5'-AGG ATG GTC ATA ATC AAA GTC GG-3'(SEQ ID NO: 19); Fragment B: #313 Mut207 5'-CCG ACT TTG ATT ATG ACC ATC CTG TTG TCG TAG CAG AGA TTA AGA GAT GGG G-3' (SEQ ID NO: 20) and #314 Mut402 5'-CGA CAA TGT CAT GGT GGT CGA AAA AAT CAT GCT GTG CTC CGT ACG-3' (SEQ ID NO: 21). Fragments A and B were mixed in equimolar ratios and subsequently the full-length fragment was amplified with the external primers: #312 Mut176 and #314 Mut402. This fragment was used in a multimerization reaction with the vector PCR fragment created with the primers #296 Y402multi 5'-TTT CGA CCA CCA TGA CAT TGT CG-3' (SEQ ID NO: 22) and #305 399Multi176 5'-TAT AGA TGC GGT TCA GCT TTC GGG-3' (SEQ ID NO: 23) on template LE399 as described above. The multimerization reaction was subsequently transformed into B. subtilis. Clones were screened for stability in the assay mentioned above. The presence of the mutations from LE493 in several clones with increased stability was confirmed by sequencing.

LE 497 was obtained in a similar manner by amplifying the LE399 encoding template with primers #312 Mut176 and #314 Mut402 and using the resulting PCR fragment in a multimerization reaction with a vector fragment obtained by PCR amplification of the LE399 template with the primers #296 Y402multi and #305 399Multi176.

Results:

Stabilization of LE399 variant at pH 4.5, 5ppm calcium incubated at 90oC

Name	LE399	LE495	LE497
Mutations	- (backbone)	K176R D207V Y402F	K176R Y402F
Stability1)	-	+	+

1) A "+" indicates significant increase in stability relative to backbone.

Claims:

1. An isolated variant of a parent alpha-amylase, wherein:

(a) the variant has at least 90% sequence identity to SEQ ID NO: 6,

(b) the variant comprises a substitution of serine at position 239 relative to the parent alpha-amylase, using the amino acid sequence of SEQ ID NO: 8 for determining position numbering, and

(c) the variant has alpha-amylase activity, wherein the variant has increased thermostability relative to the parent alpha-amylase.

2. The variant of Claim 1, wherein the variant has at least 95% sequence identity to SEQ ID NO: 6.

3. The variant of Claim 1, wherein the variant has at least 97% sequence identity to SEQ ID NO: 6.

4. The variant of Claim 1, wherein the variant has at least 99% sequence identity to SEQ ID NO: 6.

5. The variant of Claim 1, wherein the parent alpha-amylase is a *Bacillus stearothermophilus* alpha-amylase.

6. The variant of Claim 5, wherein the *Bacillus stearothermophilus* alpha-amylase is the amino acid sequence of SEQ ID NO: 6.

7. The variant of claim 1, wherein the thermostability is determined at a temperature in the range of 70-120°C, a pH in the range of 4-6, and a calcium concentration below 60 ppm

8. The variant of claim 1, wherein the thermostability is determined at pH 4.5, 90°C and 5 ppm calcium.

9. The variant of Claim 1, wherein the variant further comprises an alteration at one or more positions selected from the group consisting of 49, 60, 104, 132, 161, 170, 176, 179, 180, 181, 183, 200, 203, 204, 207, 212, 237, 250, 280, 298, 318, 374, 385, 393, 402, 406, 427, 430, 440,

444, 447, and 482, wherein the alteration(s) are independently selected from an insertion, a deletion, or a substitution.

10. A composition comprising the variant of Claim 1 and (i) another alpha-amylase; or (ii) one or more enzymes selected from the group consisting of glucoamylase, phytase, and pullalanase.

11. An isolated variant of a parent alpha-amylase, wherein:

(a) the variant has an amino acid sequence with 1-15 point alteration(s) relative to the parent alpha-amylase shown in SEQ ID NO: 6, wherein

(i) the 1-15 point alteration(s) are independently selected from an insertion, a deletion, or a substitution, and

(i) the 1-15 point alteration(s) include a substitution of serine at position 239, and

(b) the parent alpha-amylase has at least 90% sequence identity to SEQ ID NO: 6, and

(c) the amino acid sequence of SEQ ID NO: 8 is used for determining position numbering, and

(d) the variant has alpha-amylase activity, and wherein the variant has increased thermostability relative to the parent alpha-amylase.

12. The variant of Claim 11, wherein the variant has 1 alteration relative to the parent alpha-amylase in SEQ ID NO: 6 which is the substitution of the amino acid at position 239 using SEQ ID NO: 8 for determining position numbering.

13. The variant of Claim 11, wherein the thermostability is determined at a temperature in the range of 70-120°C, a pH in the range of 4-6, and a calcium concentration below 60 ppm

14. The variant of Claim 11, wherein the thermostability is determined at pH 4.5, 90°C and 5 ppm calcium

15. The variant of Claim 11, wherein the parent alpha-amylase has at least 95% sequence identity to SEQ ID NO: 6.

16. The variant of Claim 11, wherein the parent alpha-amylase has at least 99% sequence identity to SEQ ID NO: 6.

17. The variant of Claim 11, wherein one or more alteration(s) are at a position selected from the group consisting of 49, 60, 104, 132, 161, 170, 176, 179, 180, 181, 183, 200, 203, 204, 207, 212, 237, 250, 280, 298, 318, 374, 385, 393, 402, 406, 427, 430, 440, 444, 447, and 482.

18. A composition comprising the variant of Claim 11 and (i) another alpha-amylase or (ii) one or more enzymes selected from the group consisting of glucoamylase, phytase and pullulanase.

19. An isolated variant of a *Bacillus stearothermophilus* parent alpha-amylase shown in SEQ ID NO: 6, wherein the variant consists of a substitution of serine at position 239 with a different amino acid, using the amino acid sequence of SEQ ID NO: 8 for determining position numbering, and wherein the variant has alpha-amylase activity, wherein the variant has increased thermostability relative to the parent alpha-amylase.

20. The variant of claim 19, wherein the thermostability is determined at a temperature in the range of 70-120°C, a pH in the range of 4-6, and a calcium concentration below 60 ppm

21. The variant of claim 19, wherein the thermostability is determined at pH 4.5, 90°C and 5 ppm calcium

22. Use of a variant of claim 1 for starch liquefaction.

23. Use of a variant of claim 1 for ethanol production.

	1				50
1	HHNGTNGTMM	QYFEWHLPND	GNHWNRLRDD	ASNLRNRGIT	AIWIPPAWKG
2	HHNGTNGTMM	QYFEWYLPND	GNHWNRLRDD	AANLKSKGIT	AVWIPPAWKG
3	....VNGTLM	QYFEWYTPND	GQHWKRLQND	AEHLSDIGIT	AVWIPPAYKG
4	..ANLNGTLM	QYFEWYMPND	GQHWRRLQND	SAYLAEHGIT	AVWIPPAYKG
5	.AAPFNGTMM	QYFEWYLPDD	GTLWTKVANE	ANNLSSLGIT	ALWLPPAYKG
	51				100
1	TSQNDVGYGA	YDLYDLGEFN	QKGTVRTKYG	TRSQLESAIH	ALKNNGVQVY
2	TSQNDVGYGA	YDLYDLGEFN	QKGTVRTKYG	TRNQLQAAVT	SLKNNGIQVY
3	LSQSDNGYGP	YDLYDLGEFQ	QKGTVRTKYG	TKSELQDAIG	SLHSRNVQVY
4	TSQADVGYGA	YDLYDLGEFH	QKGTVRTKYG	TKGELQSAIK	SLHSRDINVY
5	TSRSDVGYGV	YDLYDLGEFN	QKGTVRTKYG	TKAQYLQAIQ	AAHAAGMQVY
	101				150
1	GDVVMNHKGG	ADATENVLAV	EVNPNNRNQE	ISGDYTIEAW	TKFDFPGRGN
2	GDVVMNHKGG	ADGTEIVNAV	EVNRSNRNQE	TSGEYAIEAW	TKFDFPGRGN
3	GDVVLNHKAG	ADATEDVTAV	EVNPANRNQE	TSEEYQIKAW	TDFRFPGRGN
4	GDVVINHKGG	ADATEDVTAV	EVDPADRNRV	ISGEHLIKAW	THFHFPGRGS
5	ADVVFDHKGG	ADGTEWVDAV	EVNPSDRNQE	ISGTYQIQAW	TKFDFPGRGN
	151				200
1	TYSDFKWRWY	HFDGVDWDQS	RQFQNRIYKF	RGDGKAWDWE	VDSENGNYDY
2	NHSSFKWRWY	HFDGTDWDQS	RQLQNKIYKF	RGTGKAWDWE	VDTENGNYDY
3	TYSDFKWHWY	HFDGADWDES	RKI.SRIFKF	RGEGKAWDWE	VSSENGNYDY
4	TYSDFKWHWY	HFDGTDWDES	RKL.NRIYKF	..QGKAWDWE	VSNENGNYDY
5	TYSSFKWRWY	HFDGVDWDES	RKL.SRIYKF	RGIGKAWDWE	VDTENGNYDY

Fig. 1

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     201                                                  250
1    LMYADVDMDH PEVVNELRRW GEWYTNTLNL DGFRIDAVKH IKYSFTRDWL
2    LMYADVDMDH PEVIHELRNW GVWYTNTLNL DGFRIDAVKH IKYSFTRDWL
3    LMYADVDYDH PDVVAETKKW GIWYANELSL DGFRIDAAKH IKFSFLRDWV
4    LMYADIDYDH PDVAAEIKRW GTWYANELQL DGFRLDAVKH IKFSFLRDWV
5    LMYADLDMDH PEVVTELKNW GKWYVNTTNI DGFRLDAVKH IKFSFFPDWL

     251                                                  300
1    THVRNATGKE MFAVAEFWKN DLGALENYLN KTNWNHSVFD VPLHYNLYNA
2    THVRNTTGKP MFAVAEFWKN DLGAIENYLN KTSWNHSAFD VPLHYNLYNA
3    QAVRQATGKE MFTVAEYWQN NAGKLENYLN KTSFNQSVFD VPLHFNLQAA
4    NHVREKTGKE MFTVAEYWQN DLGALENYLN KTNFNHSVFD VPLHYQFHAA
5    SYVRSQTGKP LFTVGEYWSY DINKLHNYIT KTDGTMSLFD APLHNKFYTA

     301                                                  350
1    SNSGGNYDMA KLLNGTVVQK HPMHAVTFVD NHDSQPGESL ESFVQEWFKP
2    SNSGGYYDMR NILNGSVVQK HPTHAVTFVD NHDSQPGEAL ESFVQQWFKP
3    SSQGGGYDMR RLLDGTVVSR HPEKAVTFVE NHDTQPGQSL ESTVQTWFKP
4    STQGGGYDMR KLLNGTVVSK HPLKSVTFVD NHDTQPGQSL ESTVQTWFKP
5    SKSGGAFDMR TLMTNTLMKD QPTLAVTFVD NHDTEPGQAL QSWVDPWFKP

     351                                                  400
1    LAYALILTRE QGYPSVFYGD YYGIPTHS.. .VPAMKAKID PILEARQNFA
2    LAYALVLTRE QGYPSVFYGD YYGIPTHG.. .VPAMKSKID PLLQARQTFA
3    LAYAFILTRE SGYPQVFYGD MYGTKGTSPK EIPSLKDNIE PILKARKEYA
4    LAYAFILTRE SGYPQVFYGD MYGTKGDSQR EIPALKHKIE PILKARKQYA
5    LAYAFILTRQ EGYPCVFYGD YYGIPQYN.. .IPSLKSKID PLLIARRDYA

     401                                                  450
1    YGTQHDYFDH HNIIGWTREG NTTHPNSGLA TIMSDGPGGE KWMYVGQNKA
2    YGTQHDYFDH HDIIGWTREG NSSHPNSGLA TIMSDGPGGN KWMYVGKNKA
3    YGPQHDYIDH PDVIGWTREG DSSAAKSGLA ALITDGPGGS KRMYAGLKNA
4    YGAQHDYFDH HDIVGWTREG DSSVANSGLA ALITDGPGGA KRMYVGRQNA
5    YGTQHDYLDH SDIIGWTREG GTEKPGSGLA ALITDGPGGS KWMYVGKQHA
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Fig. 1 (continued)

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    451                                                     500
1   GQVWHDITGN KPGTVTINAD GWANFSVNGG SVSIWVKR.. ..........
2   GQVWRDITGN RTGTVTINAD GWGNFSVNGG SVSVWVKQ.. ..........
3   GETWYDITGN RSDTVKIGSD GWGEFHVNDG SVSIYVQ... ..........
4   GETWHDITGN RSEPVVINSE GWGEFHVNGG SVSIYVQR.. ..........
5   GKVFYDLTGN RSDTVTINSD GWGEFKVNGG SVSVWVPRKT TVSTIARPIT

    501                 519
1   .......... .........
2   .......... .........
3   .......... .........
4   .......... .........
5   TRPWTGEFVR WTEPRLVAW
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Fig. 1 (continued)